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CONTRACT NO.: DAMD17-90-C-0037

TITLE: Host Factors Contributing to Disability Following Sulfur Mustard Exposure

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REPORT DATE: January 31, 1995

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
FORT DETRICK
Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE 01/31/95

3. REPORT TYPE AND DATES COVERED Final Report 03/30/90-12/31/94

4. TITLE AND SUBTITLE
Host Factors Contributing to Disability Following Sulfur Mustard Exposure

5. FUNDING NUMBERS
DAMD17-90-C-0037

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U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
Fort Detrick
Frederick, MD 21702, 5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*
Cytokines in SM lesions. We demonstrated the mRNAs of four major cytokines in developing and healing rabbit SM lesions: Interleukin 1 (beta), (IL-1 (beta)), neutrophil attractant/activation protein-1 (NAP-1 or IL-8), monocyte chemoattractant (activating) protein 1 (MCP-1), and the chemokine GRO (a growth factor and chemoattractant for granulocytes). The macrophages and activated fibroblasts in the lesions contained the mRNA of all four cytokines, with the highest amounts in the peak lesions and decreased amounts during healing. Granulocytes contained the mRNA of IL-1 (beta) and NAP-1. In the epithelial cells of hair follicles, GRO mRNA was up-regulated as early as 1 hour after the the application of sulfur mustard and remained high during the healing process. In SM lesions (but not in normal skin), surface epithelial cells and/or hair follicle epithelial cells contained the mRNA of NAP-1, MCP-1 and GRO. Evidently,
(continued on next page)

14. SUBJECT TERMS
Sulfur Mustard: bis(2-chloroethyl)sulfide (SM); Monocyte Chemoattractant (Activating) Protein-1 (MCP-1); Neutrophil Attractant/Activation Protein-1 (NAP-1); Interleukin 1 (IL-1); (continued)

15. NUMBER OF PAGES 78

16. PRICE CODE N.A.

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
unclassified	unclassified	unclassified	unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Report Documentation Page (page 2)

13. ABSTRACT (continued)

SM induces such epithelial cells to produce the mRNA of these chemotactic/activating cytokines, which, in turn, chemoattract polymorphonuclear or mononuclear phagocytes and locally activate the fibroblasts. These three cell types then produce more cytokines which are major participants in the inflammatory and healing processes. The abundance of GRO mRNA in hair follicle epithelial cells suggests that main function of this chemokine is re-epithelialization. In contrast, the main functions of NAP-1 and MCP-1 are probably the chemotaxis and activation of phagocytes.

Hydrogen peroxide in SM lesions. We have developed a histochemical test for the production of H_2O_2 in tissue sections of SM lesions. Intact granulocytes, as well as those recently dead in vivo, were major producers of H_2O_2. Cells in the macrophage-fibroblast group also produced it in lesser amounts. The H_2O_2 produced production that we demonstrated in the granulocytes (found in tissue sections) was not from their main oxygen-consuming metabolic pathway: the flavine-requiring NADPH oxidase is a very labile enzyme that does not survive cold paraformaldehyde fixation. The H_2O_2 was produced by more stable oxidases that still need to be specifically identified. No tissue destruction was seen adjacent to the cells producing H_2O_2, apparently because antioxidants in the tissues and in the extravasated serum prevented tissue damage by the H_2O_2.

Effect of certain inflammatory inhibitors on SM lesions. Interleukin 1 receptor antagonist protein (IL-1ra), soluble IL-1 receptor, soluble TNF receptor, leukotriene B_4 and phospholipase inhibitors, and a few other inflammatory inhibitors were each injected into SM lesions. The purpose of these experiments was to discover new therapeutic agents for the treatment of SM burns. Although some of these inhibitors had slight gross or histologic effects, none appreciably hastened the healing of the SM lesions. To find new effective therapeutic agents is looking for "a needle in a haystack." We are glad that we made the effort because the rewards would have been so great and there were so many new types of agents to evaluate --- especially those that inhibit various cytokines.

14. SUBJECT TERMS (CONT'D)

Interleukin 8 (IL-8) (same as NAP-1); GRO - A member of the CXC subfamily of chemokines that promotes the multiplication of cells. Histochemistry of H_2O_2 production. Cytokine inhibitors.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

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January 31, 1995
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Arthur M. Dannenberg Jr.
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SUMMARY

CYTOKINES IN SM LESIONS.

Cytokines are autocrine and paracrine protein hormones produced by cells in response to specific and nonspecific stimuli. They play a major role in both acute and chronic inflammatory processes, including those produced by sulfur mustard (SM). Understanding of the role of cytokines in SM lesions should lead to better therapy because various cytokine activators and inhibitors are becoming available.

In situ hybridization of the mRNA of various cytokines with radiolabeled antisense RNA probes enables us to visualize under the microscope which cells in tissue sections of SM lesions are producing which type of cytokine. This technique, therefore, demonstrates cell function histologically, even though the cells are no longer alive at the time of analysis.

We demonstrated the mRNAs of four major cytokines in developing and healing rabbit SM lesions: Interleukin 1 (beta), (IL-1 (beta)), neutrophil attractant/-activation protein-1 (NAP-1 or IL-8), monocyte chemoattractant (activating) protein 1 (MCP-1), and the chemokine GRO (a growth factor and chemoattractant for granulocytes). The macrophages and activated fibroblasts in the lesions contained the mRNA of all four cytokines, with the highest amounts in the peak lesions and decreased amounts during healing. Granulocytes contained the mRNA of IL-1 (beta) and NAP-1. In the epithelial cells of hair follicles, GRO mRNA was up-regulated as early as 1 hour after the application of sulfur mustard and remained high during the healing process.

In SM lesions (but not in normal skin), surface epithelial cells and/or hair follicle epithelial cells contained the mRNA of NAP-1, MCP-1 and GRO. Evidently, SM induces such epithelial cells to produce the mRNA of these chemotactic/activating cytokines, which, in turn, chemoattract polymorphonuclear or mononuclear phagocytes and locally activate the fibroblasts. These three cell types then produce more cytokines which are major participants in the inflammatory and healing processes. The abundance of GRO mRNA in hair follicle epithelial cells suggests that main function of this chemokine is re-epithelialization. In contrast, the main functions of NAP-1 and MCP-1 are probably the chemotaxis and activation of phagocytes.

HYDROGEN PEROXIDE IN SM LESIONS.

We have developed a histochemical test for the production of H_2O_2 in tissue sections of SM lesions. Intact granulocytes, as well as those recently dead in vivo, were major producers of H_2O_2. Cells in the macrophage-fibroblast group also produced it in lesser amounts.

Summary (continued)

The H_2O_2 produced production that we demonstrated in the granulocytes (found in tissue sections) was not from their main oxygen-consuming metabolic pathway: the flavine-requiring NADPH oxidase is a very labile enzyme that does not survive cold paraformaldehyde fixation. The H_2O_2 was produced by more stable oxidases that still need to be specifically identified.

No tissue destruction was seen adjacent to the cells producing H_2O_2, apparently because antioxidants in the tissues and in the extravasated serum prevented tissue damage by the H_2O_2

EFFECT OF CERTAIN INFLAMMATORY INHIBITORS ON SM LESIONS

Interleukin 1 receptor antagonist protein (IL-1ra), soluble IL-1 receptor, soluble TNF receptor, leukotriene B_4 and phospholipase inhibitors, and a few other inflammatory inhibitors were each injected into SM lesions. The purpose of these experiments was to discover new therapeutic agents for the treatment of SM burns.

Although some of these inhibitors had slight gross or histologic effects, none appreciably hastened the healing of the SM lesions. To find new effective therapeutic agents is looking for "a needle in a haystack." We are glad that we made the effort because the rewards would have been so great and there were so many new types of agents to evaluate --- especially those that inhibit various cytokines.

GENERAL INTRODUCTION

These studies began when cytokines were known to be major mediators of the cellular immune reaction, but little was known about their role in non-immune inflammatory processes. Over the past five years, however, this picture changed: Cytokines are now known to mediate all inflammmatory reactions. In addition, as briefly reviewed in the Discussion of Chapter 1, almost every inflammatory mediator directly or indirectly has an effect on cytokine production and often vice versa.

In carrying out this Contract, we have spent the major part of our effort on visualizing (by in situ hybridization) the mRNAs of various cytokines in cells within developing, peak and healing dermal sulfur mustard (SM) lesions. In situ hybridization of such mRNAs with ^{35}S-antisense riboprobes enables us to visualize what a given cell can produce, even though in a tissue section the cell is no longer alive. The amount of cytokine mRNA in a cell should reflect the amount of the cytokine that the cell can produce. Apparently, because cytokine proteins are short-lived and usually in low concentrations, we could not visualize such proteins in tissue sections of SM lesions by immunohistochemical techniques.

In addition to studying cytokines, we studied histochemically in SM lesions the production of H_2O_2. Finally, we evaluated the effects of various cytokine inhibitory agents and other anti-inflammatory agents on the development and healing of SM lesions.

These studies provide basic information on the role of cytokines in the development and healing of SM lesions. Although none of the cytokine inhibitors listed in this report appreciably affected the course of SM lesions, many more experiments, especially those involving combinations of several inhibitors, should be performed. The appropriate modulation of cytokine action could some day be of great use in stopping the progression and hastening the healing of SM lesions.

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Chapter 1

The Cytokines NAP-1 (IL-8), MCP-1, IL-1 (beta), and GRO in Dermal Inflammatory Lesions Produced by the Chemical Irritant Sulfur Mustard

ABSTRACT

Developing and healing dermal inflammatory lesions were produced in rabbits by the topical application of dilute sulfur mustard (SM), the military vesicant. In tissue sections of such lesions, cells containing the mRNA of important cytokines were identified with in situ hybridization techniques. These cytokines were neutrophil attractant/activation protein-1 (NAP-1 or IL-8), monocyte chemoattractant (activating) protein 1 (MCP-1), interleukin 1 (beta) (IL-1 (beta)), and GRO (a growth factor and chemokine).

Macrophages and activated fibroblasts contained the mRNA of all four cytokines, and granulocytes contained the mRNA of IL-1 (beta) and NAP-1. More cytokine-producing cells were present in lesions when they were at peak size than when they were healing. Granulocytes emigrated from the bloodstream, passed through the lesions, and were the major constituent of the protective crust. This sequence correlated with the distribution of cells able to produce NAP-1: The granulocytes and macrophage/fibroblasts that contained messenger RNA for this granulocyte chemoattractant were found mainly in the upper part of the dermis. In contrast, cells containing the mRNA for the monocyte chemoattractant, MCP-1, predominated in middle and deep parts of the dermis until 6 days, when the lesions were almost healed.

SM stimulated hair follicle epithelial cells to up-regulate GRO mRNA and, to a lesser degree, NAP-1 mRNA. Apparently, the irritation produced by SM directly or indirectly induces such epithelial cells to manufacture these growth factors. In the rabbit, hair follicles are known to be the main source of new epithelial cells after the covering epithelium has been destroyed. Therefore, GRO seems to be a major autocrine-paracrine stimulus for such repair.

Abbreviations

SM	-	Sulfur mustard: bis(2-chloroethyl)sulfide

GM-CSF	-	Granulocyte-Macrophage Colony Stimulating Factor
GRO	-	A member of the CXC subfamily of chemokines that promotes the multiplication of cells.
IFN (gamma)	-	Interferon-gamma
IL- 1	-	Interleukin 1
IL-8	-	Interleukin 8 (same as NAP-1) - - a CXC chemokine
MCP-1	-	Monocyte Chemoattractant (Activating) Protein-1 - - a CC chemokine
NAP-1	-	Neutrophil Attractant/Activation Protein-1 (same as IL-8) - - a CXC chemokine
TGF (beta)	-	Transforming Growth Factor (beta)
TNF (alpha)	-	Tumor Necrosis Factor (alpha)

EDTA	-	Ethylenediamine tetraacetate
DEPC	-	Diethylpyrocarbonate
PAF	-	Platelet Activating Factor
PBS	-	Phosphate-buffered saline solution
PGI_2	-	Prostaglandin I_2 (prostacyclin)
SSC	-	Sodium chloride--sodium citrate solution

INTRODUCTION

We have spent many years elucidating the inflammatory processes of skin lesions produced in rabbits by the chemical irritant sulfur mustard. The rabbit was chosen because a single rabbit has enough skin surface to contain simultaneously both developing and healing lesions (produced by applying the irritant at different times). Our previous studies on such lesions have concerned the leukocyte composition (1,2), the serum turnover (3), the early mediators (histamine, prostaglandin E_2, and plasminogen activators) (4), and the remodeling associated with healing (by collagenase, stromelysin and their inhibitors) (5,6). These studies and others are reviewed in reference 7.

The present study concerns the role of some of the major cytokines in this model of chemical-induced inflammation. Few studies have been made on the cytokines of rabbit inflammatory lesions, and none have been made on dermal lesions produced by sulfur mustard in this species.

Cytokines are important mediators of all inflammatory processes: those caused by irritants (8-12) as well as those caused by antigens (11,12). A network of cytokines exists in which synergism and up- and down-regulation by each other take place (13-16). Each cytokine works through its own receptor (16-25), and the resulting cell response is influenced by both the number and type of receptors, as well as the concentration of the cytokine itself.

Cytokines are short-lived and can only rarely be detected in tissue sections. However, cells that can produce cytokines can be visualized with labeled cDNA or antisense RNA radiolabeled probes, which hybridize with specific cytokine mRNA within the cells in tissue sections (26,27). Sense RNA probes serve as non-hybridizing controls for antisense RNA probes. Since both probes would bind to double-stranded nuclear DNA, negative results with sense probes also distinguish mRNA binding from DNA binding.

Unfortunately, there are relatively few recombinant plasmids containing cDNA inserts of rabbit cytokines. However, several important ones were available and were used for the in situ hybridization studies reported herein. We found a good correlation between (a) the cells containing mRNAs of major chemokines and (b) the distribution of cells that respond to these chemokines. We also found that hair follicle cells contained high levels of GRO mRNA. Hair follicle cells are the major source of the new epithelium that replaces the epithelium killed by the sulfur mustard. Therefore, in the rabbit, GRO seems to be a major autocrine-paracrine stimulus for such repair.

MATERIALS AND METHODS

Preparation of ^{35}S-labeled RNA probes

The molecular biological techniques for these procedures are described in references 28 and 29.

Recombinant Bluescript plasmids containing cDNA for rabbit NAP-1 (IL-8) (30), rabbit MCP-1 (30), and rabbit GRO were provided by our co-author, Teizo Yoshimura. The rabbit GRO cDNA was cloned from the rabbit spleen cell cDNA library described in reference 30. The cDNA sequence matches the cDNA sequence expected from the amino acid composition of rabbit GRO published in reference 31.

Recombinant Okayama plasmids containing cDNA for rabbit IL-1 (beta) were provided by Masaru Yoshinaga (First Department of Pathology, Kumamoto University, Kumamoto, Japan (32,33)). The cDNAs of these plasmids were excised and inserted into pBluescript (Stratagene, 1109 N. Torrey Pines Rd., LaJolla, CA). Then, E. coli (strain L-1 Blue) was transfected and grown to expand the new recombinant plasmid.

From the pBluescript or pGEM recombinant plasmids, cytokine cDNA can be linearized with the appropriate restriction enzymes, and antisense and sense ^{35}S-riboprobes can be prepared. ^{35}S-alpha-UTP (Dupont/NEN Research Products, Boston MA) and the TransProbe T kit (Pharmacia/LKB Biotechnology, Piscataway, NJ) were used. Briefly, ^{35}S-antisense RNA probes (and ^{35}S-sense negative control probes) were produced by transcription with T7 or T3 DNA-dependent RNA polymerase - - one for the antisense probe and one for the sense probe, depending on the direction of the plasmid insert. The template DNA was removed by digestion with DNase. After ethanol precipitation and washing, each riboprobe was redissolved in 20 ul 10 mM Tris-HCl/1 mM EDTA, and 50 ul ethanol was added. These ^{35}S-labeled riboprobes were then stored at -80°C and used for in situ hybridizations over the next 4 months.

<u>Skin lesions produced by dilute sulfur mustard (SM)</u>

SM (10 ul of 1.0% SM in methylene chloride) was applied topically to the flanks of 2.5-3.0 kg female New Zealand white rabbits after the hair was removed with electric clippers. The applications were staggered, so that at the time of sacrifice, each rabbit had 16 SM lesions: two for each of the following durations: 1, 2, 4, and 8 hours, and 1, 2, 3, and 6 days. Two rabbits were usually used in each experiment. The rabbits were euthanized by an intravenous injection of pentobarbital (65 mg/ml, 2.2 to 2.8 ml). The skin of the flanks containing the lesions was immediately removed, wrapped in Saran Wrap, and chilled under cracked ice. Then, the SM lesions were bisected, removed from the cold flank skin, shaken for 4 to 5 hr in cold (4°C) 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2), and then shaken overnight at 4°C in 20% sucrose in phosphate-buffered saline (PBS) (0.01 M sodium phosphate and 0.15 M NaCl, pH 7.2).

<u>Preparation of fixed frozen tissue sections</u>

On the next day, the lesions were shaken for 2 hr at 4°C in PBS containing 5% glycerol and 20% sucrose. Then, they were placed into molds (Cryomolds, Miles, Inc., Elkhart, IN) containing Tissue-Tek O.C.T. Embedding Compound (Miles), frozen in liquid nitrogen, wrapped in Parafilm (American National Can Co., Greenwich, CT), and stored at -80°C.

DEPC-treated water was used for all reagents (28,29). DEPC water is distilled (or deionized) water, treated with 0.1% diethylpyrocarbonate at 23°C for at least 12 hr to inactivate RNases, and autoclaved for 15 min to remove the DEPC.

The frozen specimens were cut in a cryostat at 6 um, placed on silane-coated microscope slides (Superfrost/Plus slides, Fisher Scientific, Pittsburgh, PA), and immediately dried with a cool hair dryer. The sections were fixed again with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 10 to 20 min at

23°C, rinsed in turn with 3X PBS, 1X PBS, and 1X PBS for 5 min each, rinsed briefly with DEPC-treated water, dehydrated in ascending concentrations of ethanol (30, 60, 80 and 95%) for 3 min each, dried with the hair dryer, and stored at -80°C in a slide box containing desiccant and sealed with tape. Slides stored under these conditions can be used for in situ hybridization for several months without significant loss of the hybridizing mRNAs that we studied. Fixed-frozen sections seemed preferable to unfixed sections because of better preservation of mRNA and tissue structure in general.

<u>In situ hybridization of cytokine mRNA in the tissue sections</u> (34-36).

The tissue sections were digested at 37°C for 30 min in proteinase K (1 ug/ml in 100 mM Tris-HCl containing 50 mM EDTA at pH 8), following which, they were fixed again for 10 min in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, to stabilize cellular mRNA within the proteolyzed matrix. Then, they were washed in PBS and DEPC-treated water as just described. The free amino groups were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min at 23°C, and the sections rinsed briefly in DEPC-treated water and dried again with the hair dryer.

For hybridization, the dried sections were overlaid with 10 ul of hybridization solution consisting of the probe (heat-denatured just before use at 80° to 95°C for 3 min), formamide (50% final concentration), NaCl (300 mM), Tris-HCl, (20 mM, pH 8.0), EDTA (5 mM), Denhardt's solution (1X), dextran sulfate (10%), dithiothreitol (DTT) (10 mM), and yeast tRNA (400 ug/ml). The probe in this 10 ul hybridization solution contained 200,000 to 600,000 cpm of ^{35}S. The tissue sections were covered with silicone-coated glass coverslips and sealed around the edges with rubber cement. [The coverslips were previously baked at 150°C for 18 hr to inactivate RNases.] The sections were then hybridized for 17 to 20 hr at 45°C in a moist chamber.

The unhybridized probe was washed from the slides in a solution of 50% formamide, 2X SSC, 10 mM DTT, and 1 mM EDTA for 30 min at 45°C, following which the tissue sections were washed twice, briefly, with 2X SSC containing 10 mM DTT, and then digested with RNase A (20 ug/ml) for 30 min at 37°C. [1X SSC is 0.15 M NaCl and 0.015 M sodium citrate in DEPC water at pH 7.0 (28,29).] They were washed two more times with a solution of 50% formamide, 2X SSC, 10 mM DTT and 1 mM EDTA at 45°C for 30 min, each. In some experiments, a higher stringency wash of 0.2X SSC with 10 mM DTT was used. Finally, the slides were briefly rinsed with a solution of 2X SSC and 1 mM DTT, dehydrated through graded ethanols containing 300 mM ammonium acetate, and dried with the hair dryer.

For autoradiography, the slides were dipped into Kodak NTB-2 emulsion diluted with equal parts of 600 mM ammonium acetate and exposed in the dark at 4°C for 7-21 days. They were then developed and counterstained with Giemsa (37).

In situ hybridizations for cytokine mRNAs were performed with antisense probes (complementary to cellular mRNA). As negative controls, duplicate tissue sections were also hybridized with sense RNAs (homologous to cellular mRNA). Such positive and negative controls were included in each run.

Counting the ^{35}S-labeled cells

In Giemsa-stained tissue sections of SM lesions, labeled and unlabeled cells in four groups were counted microscopically with a 40X objective lens: (a) mononuclears (mainly macrophages and fibroblasts with some medium to large lymphocytes (b) granulocytes (mostly eosinophilic heterophils which, in the rabbit, are equivalent to human neutrophils), (c) epidermal cells, and (d) the epithelial cells of hair follicles. A 1.0 cm^2 ocular grid that measured 0.25 mm across the field of the 40X objective lens was used, and all labeled and unlabeled cells in 40 (rather evenly spaced) grid areas were counted in each of the upper, middle and deep areas of the dermis. We could not always distinguish macrophages from fibroblasts in tissue sections because macrophages can be elongated, and activated fibroblasts can be "short and plump." We therefore counted them together as one group. They both are mesenchyme cells, and both seem to produce the same types of cytokines.

The inflammatory lesions are rather thick and quite edematous during the first few days (1). The tissue sections (cut vertically) measure 1 to 2 mm. Our ocular grid covers an area of only 0.25 X 0.25 mm in the tissue section. Therefore, one can easily select representative upper, middle and lower areas for counting, although there is no morphological demarcation between them.

We counted all of the epidermal cells and hair follicle cells in the tissue section (about 1 cm in length). With a 40X objective lens, these cells usually did not fill the entire grid. Therefore, the mm^2 areas reported in Table 1 really represent 1.0 mm lengths, i.e., we did not multiply by 5 if the epithelial cells only filled one-fifth of the grid.

RESULTS

Development and healing of sulfur mustard (SM) lesions in rabbits

Details of the gross and microscopic events as these lesions progress and regress were published in references 1, 2, 5, and 38. In brief, erythema and edema begin as early as 1 hr after the topical application of SM. At 1 day, the epidermis is dying or already dead, and crust (or scab) formation begins. The lesions reach peak size in 1 or 2 days. By 3 days, healing has begun. Edema is much reduced, and often a prominent crust is present with epidermal cells beginning to migrate under the crust. The lesions healed in 6 to 10 days.

The major cells participating in the SM lesions are macrophages, granulocytes and activated fibroblasts (2). In this sterile chemical-induced inflammatory process, macrophages are the major infiltrating cell in the acute stages at 1 and 2 days and remain prominent during the healing process (2). Granulocytes in SM lesions emigrate from the microvasculature (venules) and migrate to the surface to form the protective crust (1,2). During healing (on day 6), very few granulocytes remained in the middle and lower dermis (Table 2). Many fibroblasts are activated in peak (1-day) lesions, possibly due to locally released cytokines as well as the ingestion of extravasated serum proteins (2,3). However, there are many more activated fibroblasts in healing (6-day) lesions (2), where they play a major role in the remodeling process (5).

Overview of our findings on cytokine mRNAs in the SM lesions

Tables 1, 2 and 3 are a summary of our findings. For each cytokine, cells were labeled only with the antisense RNA probes, not with the sense RNA probes. We counted upper, middle and lower areas of the dermis separately and added them together to produce the totals presented in Table 1. For NAP-1 and MCP-1, these areas are listed separately in Table 2.

In general, the number of cells labeled for the mRNAs of NAP-1, MCP-1, IL-1 (beta), and GRO increased during the first day and decreased during healing (Table 1). The number of macrophage/fibroblasts labeled for NAP-1 mRNAs was higher than the number of granulocytes so labeled, but the two groups were about equally labeled for IL-1 mRNA. The granulocytes contained no MCP-1 mRNA and very little GRO mRNA.

In SM lesions (but not in normal skin) large numbers of hair follicle cells were often labeled for GRO mRNA and were occasionally labeled for NAP-1 mRNA (Table 1). These follicle cells were rarely labeled for MCP-1 mRNA and never labeled for IL-1 (beta) mRNA. A few epidermal cells were labeled for NAP-1 mRNA and GRO mRNA, but not for IL-1 (beta) mRNA and MCP-1 mRNA.

We interpret these findings in the following manner: SM kills the epidermis in the area in which it is applied. At the edge of the injury, a few viable epidermal cells may be stimulated by the inflammatory process, but most of the stimulation occurs in the epithelial cells of the hair follicles. Only a few hair follicle cells were killed by the SM. Most of them remained viable, and their proliferation and migration are the main mechanisms by which the defect in the epidermis is repaired. The presence of large quantities of GRO mRNA in hair follicle cells (and macrophage/fibroblasts) suggests that this growth factor is a major autocrine-paracrine stimulus to re-epithelialization.

Early mediators of inflammation

As early as 2 hr after the application of SM, MCP-1 and GRO mRNAs were

increased in the macrophage/fibroblast group (Table 1). NAP-1 and IL-1 (beta) mRNAs were upregulated more slowly in these cells. When sections of lesions from the same rabbit were hybridized, greater numbers of macrophage/fibroblasts were labeled for MCP-1 and GRO than were labeled NAP-1 and IL-1 (beta). This finding suggests that the MCP-1 and GRO play major roles. An alterative interpretation would be that the probes for MCP-1 and GRO hybridize more efficiently than the probes for NAP-1 and IL-1 (beta).

Rabbit-to-rabbit variations

The data shown in Tables 1 and 2 are from SM lesions of different ages on a given rabbit, i.e., developing and healing lesions from a single rabbit pelt were hybridized with the same probe at the same time. In order to assess variations among rabbits, we repeated several time points with additional rabbits (Table 3). These rabbit-to-rabbit variations were about the same as we found in other studies on sulfur mustard lesions (2).

Distribution of cells containing the mRNA of these cytokines

Throughout the course of the SM lesion, NAP-1 mRNA was present mostly in the upper dermis, more frequently in mononuclear cells, but also in numerous granulocytes (Table 2). MCP-1 mRNA was more evenly distributed throughout the dermis, with a tendency for the largest population of the labeled cells to occur in the macrophages and fibroblasts of the mid-dermis until day 6, when the lesions were nearly healed. Granulocytes did not contain MCP-1 mRNA. In general, the distribution of cells containing NAP-1 mRNA matches the distribution of granulocytes found in the lesions and suggests that this chemokine plays a major role in attracting granulocytes into the lesions. The same relationship may hold for the distribution of cells containing MCP-1 mRNA and the distribution of macrophages, but it is hard to differentiate macrophages from other mononuclear cells (especially fibroblasts) in cryostat sections. [We were more successful in glycol methacrylate-embedded tissue sections (2): The number of macrophages was highest in peak lesions, and the number of fibroblasts was highest in healing lesions.] Because there are many chemotactic mediators, we could only find a regional correlation, rather than a cell-to-cell correlation between NAP-1 and MCP-1 mRNAs and the cells that they, respectively, attract.

Neutrophil attractant/activation protein 1 (NAP-1 or IL-8)

The mRNA of this member of the chemokine (39,40) family was absent in the cells of normal skin (Tables 1 and 2). An appreciable number of macrophage/fibroblasts became labeled for NAP-1 mRNA as early as 2 hr. Then, this number substantially increased, and only decreased as the lesions healed. Fewer granulocytes than macrophage/fibroblasts were labeled for NAP-1 mRNA, and the labeling was less intense in the granulocyte group. Most of these granulocytes were in the upper dermis under the crust (Table 2 and Fig 1).

SM gradually killed all of the epidermal cells in the entire 1-cm^2 central area where it was applied. Therefore, only a few epidermal cells contained NAP-1 mRNA (Table 1). On the other hand, some of the epithelial cells of a few hair follicles contained NAP-1 mRNA soon after the application of SM (Table 1). The uneven distribution of NAP-1 mRNA labeling was probably due to differences in the stages of the hair growth cycle and to variations in the penetration of SM into the different follicles. Stimulating (not damaging) concentrations would be required.

Monocyte chemoattractant (activating) protein 1 (MCP-1)

MCP-1 mRNA is upregulated early in the mononuclear cell group (Table 1 and Fig. 2). Numerous macrophages and fibroblasts contained MCP-1 mRNA as early as 2 hr after the application of SM, and the number of labeled cells remained high during the healing process. No MCP-1 mRNA seemed to be present in granulocytes or in surface keratinocytes, and only an occasional cell was labeled in the hair follicles. Vascular endothelial cells were labeled for MCP-1 mRNA as early as 4 hr.

Interleukin 1 (beta)

The number of macrophage-fibroblasts labeled for IL-1 (beta) mRNA peaked at 1 and 2 days, and then started to decline (Table 1). The IL-1 (beta) mRNA in granulocytes appeared later and was less intense than that of the macrophage/fibroblast group. None-the-less, the labeling of both cell groups followed the same pattern (Table 1). There was no appreciable labeling of epidermal and hair follicle cells for IL-1 (beta) mRNA (Table 1).

GRO: a growth factor and chemokine

GRO was originally called MGSA (melanoma growth stimulatory activity) and is closely related to MIP-2 (Macrophage Inflammatory Protein-2) (41,42). The three forms of GRO -- alpha, beta, and gamma -- are recognized by all GRO probes, except those specifically made to distinguish between the three forms (42). In the CXC subfamily of chemokines, the first two (of the four) cysteines are separated by one amino acid (16,43). [In the CC subfamily, these first two cysteines are adjacent.] Human GRO is 25 times more potent in attracting PMN than human NAP-1 (IL-8) (41).

The GRO mRNA probe labeled many hair follicle cells (Fig. 3 and Table 1) and many cells of the macrophage/fibroblast group (Table 1). The number of cells labeled for GRO mRNA increased early in the inflammatory process, and decreased slowly as the lesions healed (Table 1). This probe also labeled a moderate number of vascular endothelial cells. Relatively few epidermal cells and granulocytes were labeled. The presence of GRO mRNA in normal epidermis and in normal hair follicles suggests that GRO is a primary cytokine of epithelial cells that does not require IL-1 or TNF for its induction. The importance of GRO in the re-

epithelialization of the SM lesion was presented above, under "Overview of our findings on cytokine mRNAs in the SM lesions."

Cells labeled for cytokine mRNA in the crust

A crust (or scab) began forming on Day 1, as soon as the epidermal cells died, and was well developed from Day 3 on. It consisted mostly of dead granulocytes with some macrophages. Almost all of the cells containing cytokine mRNA were found at the base of the crust where the cells were still viable or only recently dead (Fig. 1).

Since the crust is composed mostly of granulocytes, NAP-1 mRNA and IL-1 (beta) mRNA were the major cytokine mRNAs found there. MCP-1 mRNA was not found in granulocytes, and GRO mRNA was rarely found in them.

DISCUSSION

Cytokines in general, and the cytokines we studied

Cytokines are paracrine and autocrine polypeptide hormones (or growth factors) that activate or inhibit various cell functions in sites of inflammation (16). Most of the cytokines are apparently short-lived. For this reason, immunohistochemistry techniques often, but not always (44-47), fail to demonstrate cytokine protein in tissue sections. Cytokine mRNA seems to be more stable than cytokine protein and can often be visualized in cells where the protein itself cannot be visualized.

Cytokines have mainly been studied in *in vitro* systems. The studies herein reported are among the relatively few that attempt to assess the role of cytokines *in vivo*. Interpretation of *in vivo* results, however, is complicated by the redundancy of functions among the various cytokines (13-16) and interactions with the extracellular matrix (48). For example, IL-1 (alpha), IL-1 (beta), TNF (alpha) and IL-6 have many overlapping functions (13,14), and synergism between them exists (13,49-51).

IL-1 and TNF (alpha) (primary cytokines) (9,10), histamine (9) and neuropeptides (9) upregulate cytokine production in other cells, including the local fibroblasts (8,48,52,53), macrophages (see 54), endothelial cells, keratinocytes (51), and the infiltrating granulocytes (55) (Fig. 4). Many secondary cytokines are produced, especially the chemokines, which attract more leukocytes to the site and activate them. Simultaneously, receptors (13-15,18,22) for the various cytokines are upregulated in the local cells so that they can respond to the cytokine stimulus.

Our studies have identified some of the major players in the cytokine network and have related the mRNAs of certain chemokines with the distribution of infiltrating cells present in skin lesions produced in rabbits by the chemical irritant sulfur mustard. Similar correlations between NAP-1 (IL-8) and the PMN present, and MCP-1 and the macrophages present (57), were found in various human inflammatory sites.

The efficiency of the in situ hybridization procedure is probably quite low, and specific probes probably vary in affinity for mRNA. Therefore, in a given tissue section, the mRNAs of different cytokines cannot be quantitatively compared. Our experiments were, therefore, designed so that a given rabbit (when euthanized) contained developing, peak and healing lesions. Tissue sections of all such lesions on a given rabbit were hybridized at a single time with ^{35}S-probes for a given cytokine mRNA. In this way, changes in the number of cells that contain the mRNA of that cytokine could be recognized.

The participation of local cells in the inflammatory process was also evident in these studies. Activated fibroblasts (although not always distinguishable from macrophages) are clearly active cytokine producers and therefore major players in the inflammatory process (see 57). The epithelial cells of the hair follicles and, to a lesser degree, those of the epidermis also produce chemokines, especially the chemokine called GRO. Hair follicle epithelial cells are the major source of new epidermis when it has been destroyed by physical or chemical toxicants, including sulfur mustard, especially in animals covered with hair. A brief review of the local cell sources of cytokines and other inflammatory mediators is presented in the following section.

Resident cells producing cytokines in dermal inflammation caused by irritants

Keratinocytes. Keratinocytes (including those in the hair follicles) are active participants in the inflammatory process. They evidently store some of the primary cytokines (IL-1 and TNF) and release them when irritated or injured (8,10-12,44,58-63) (Fig 4). In rabbits, we have not, however, been able to find IL-1 (beta) mRNA in the epithelial cells of the epidermis or hair follicles (Table 1). [TNF (alpha) was not studied.] Cytokine inhibitors, such as interleukin 1 receptor antagonist (IL-1ra) (64-70) are also stored in the epidermis -- apparently to inhibit the local effects of the stored IL-1 on the keratinocytes themselves (68). In addition, epidermal cells up-regulate their production of both primary and secondary cytokines upon stimulation (8-10,12,44,51,58-61,71). Our results suggest that GRO should be added to the list of primary cytokines present in the epidermis and hair follicle epithelium (Fig. 4). Its role would be to stimulate the regrowth of epithelium (from hair follicles in the rabbit) in an autocrine/paracrine fashion. GRO probably plays a similar role in human beings (72), since it seems to be stored in human epidermal cells and released when they are injured (72).

Mast cells. Mast cells are the second type of dermal cell that is quite sensitive to local irritation (73-76) (Fig 4). Not only do they release histamine and eicosanoids (4), but they also release cytokines (77-83). We have no information on mast cell cytokine mRNAs, as we could not readily identify these cells after the frozen tissue sections went through the in situ hybridization procedures.

Nerves. Irritants release neuropeptides from cutaneous nerves (84). Neuropeptides stimulate mast cells to release their mediators, and also act on the microvasculature, including endothelial cells.

Fibroblasts. Fibroblasts are the most prevalent resident cell type in all connective tissues, including those of the dermis. They are usually rather dormant, but become activated early in the inflammatory response to irritants (2, see 57). When stimulated by IL-1 and TNF, fibroblasts evidently produce cytokines and other factors, e.g., NAP-1 (IL-8)-related chemokines (85); colony stimulating factors (GM-CSF and G-CSF) (86); IL-6 (87); and collagenase and PGE_2 (88). Our studies showed that NAP-1 (IL-8), MCP-1, IL-1(beta), and GRO mRNAs were upregulated in the activated fibroblasts and macrophages present in dermal inflammatory lesions produced in rabbits by sulfur mustard. Fibroblasts, therefore, are major participants in the inflammatory response (see 57).

Endothelial cells. When endothelial cells are activated by the primary cytokines, IL-1 and TNF, they produce PGI_2 (89), NO (89), endothelin (89), adhesion molecules for leukocytes (9,10,90), thromboplastin (89), platelet activating factor (PAF) (89), plasminogen activator (89), and primary and secondary cytokines (89). Although we did not specifically study endothelial cells in SM lesions, we did observe that endothelial cells often contained GRO mRNA, frequently contained MCP-1 mRNA and, less frequently, NAP-1 and IL-1 (beta) mRNAs. The number of endothelial cells containing GRO mRNA peaked at 4 to 8 hr.

Modulation of the inflammatory process.

The local cytokines of the inflammatory process could autocatalytically enhance their production until they become systemic and cause shock and even death. Fortunately, such life-threatening effects are rare, because many local modulating factors exist: Cytokine inhibitors, (such as IL-1 receptor antagonist (IL-1ra) (64-70), soluble IL-1 receptor (sIL-1R) (91-93), soluble TNF receptor (sTNFR) (91,94,95), and other soluble cytokine receptors (93,96)); proteases that destroy cytokines; histaminases; lactoferrin (from granulocytes) (97), and enzymes that break down eicosanoids and other phlogistic substances. These anti-inflammatory factors are usually produced slightly out-of-phase with the pro-inflammatory factors so that inflammation is limited to the local area, eventually regresses, and healing occurs (58). As the SM lesions healed, we observed a decrease in the mRNAs of various cytokines. This decrease was not

pronounced, which suggests that the cytokines we studied remained rather active during the healing process.

ACKNOWLEDGMENTS

Drs. Junji Tsuruta, Teizo Yoshimura, Yasuharu Abe, and Phoebe Mounts were co-investigators in these studies.

Dr. Paul L. Hermonat, (now at the University of Arkansas for Medical Sciences, in Little Rock), and Dr. Fusao Hirata (now at Wayne State University), helped us greatly with the molecular biology performed during the initial phases of this study. Dr. Edward J. Leonard, Laboratory of Immunobiology, National Cancer Institute, helped us in several aspects of this study.

We appreciate the technical assistance of Peggy J. Pula, Kerry H. Bosley, and Lita P. Fay during these studies and the editorial assistance of Ilse M. Harrop and Dr. Paul J. Converse. Timothy H. Phelps, of the Johns Hopkins Department of Art as Applied to Medicine, drew Figure 4.

Addendum:

An up-to-date review of the histochemistry of reactive oxygen intermediates was recently published by M.J. Karnovsky: Cytochemistry and reactive oxygen species: a retrospective. J. Histochem. 102: 15-27, 1994.

Table 1:
Cell types labeled for the mRNAs of four cytokines in dermal inflammatory lesions produced by sulfur mustard

NAP-1

Cell Type	Age of SM Lesions								
	Normal	1 hr	2 hr	4 hr	8 hr	1 da	2 da	3 da	6 da
Macrophage-fibroblasts*	-	±	+	++	+++	++	+++	++	+
Granulocytes*	-	-	±	+	+	±	++	+	+
Epidermal cells	-	-	±	-	±	±	-	±	-
Hair follicle cells	-	±	++	+	++	+	±	+	±

MCP-1

Cell Type	Age of SM Lesions								
	Normal	1 hr	2 hr	4 hr	8 hr	1 da	2 da	3 da	6 da
Macrophage-fibroblasts*	±	+	+++++	+++++	+++	+++++	+++++	+++++	++++
Granulocytes*	-	-	-	-	-	-	-	-	-
Epidermal cells	-	-	-	-	-	-	-	-	-
Hair follicle cells	±	-	±	±	-	±	-	-	-

IL-1 (beta)

Cell Type	Age of SM Lesions								
	Normal	1 hr	2 hr	4 hr	8 hr	1 da	2 da	3 da	6 da
Macrophage-fibroblasts*	-	±	±	+	++	+++	+++	++	++
Granulocytes*	-	-	-	±	+	++	+++	++	++
Epidermal cells	-	-	-	-	-	-	-	-	-
Hair follicle cells	-	-	-	-	-	-	-	-	-

GRO

Cell Type	Age of SM Lesions								
	Normal	1 hr	2 hr	4 hr	8 hr	1 da	2 da	3 da	6 da
Macrophage-fibroblasts*	±	++	+++++	+++++	+++++	++++	+++	+++	+++
Granulocytes*	-	-	-	±	±	±	-	-	-
Epidermal cells	+	-	±	±	±	+	+	±	+
Hair follicle cells	++	+	+++++	+++++	+++	+++	++++	+	+++

*Cells per 3 mm^2 of tissue section: ± = 0.4 to 10 cells labeled; + = 10 to 40 cells labeled; ++ = 40 to 80 cells labeled; +++ = 80 to 160 cells labeled; ++++ = 160 to 240 cells labeled; +++++ = 240 to 350 cells labeled. See footnote of Table 2.

Representative data from single rabbits containing lesions of all ages. Bold-face figures are the means of 5 rabbits for NAP-1 and 3 rabbits for MCP-1, when additional rabbits were used to confirm results.

Table 2

Total cells and cells labeled for the mRNAs of two chemokines in SM lesions

MAP-1 mRNA in granulocytes (PMN)

	Normal skin			Peak lesions (1 & 2 day)				Healing lesions (3 & 6 day)			
Skin depth*	Total PMN/ mm^2	Labeled PMN/ mm^2	%	Day	Total PMN/ mm^2	Labeled PMN/ mm^2	%	Day	Total PMN/ mm^2	Labeled PMN/ mm^2	%
Upper	3	0	0	1 da	243	1.2	0.5	3 da	703	25	3.6
				2 da	730	76	10.4	6 da	415	12	2.9
Middle	1	0	0	1 da	99	0.4	0.4	3 da	--	--	---
				2 da	627	10	1.6	6 da	33	0	0
Deep	1	0	0	1 da	60	0	0	3 da	--	--	---
				2 da	833	5	0.6	6 da	13	0	0

MAP-1 mRNA in macrophages/fibroblasts (MN)

	Normal skin			Peak lesions (1 & 2 day)				Healing lesions (3 & 6 day)			
Skin depth*	Total MN/ mm^2	Labeled MN/ mm^2	%	Day	Total MN/ mm^2	Labeled MN/ mm^2	%	Day	Total MN/ mm^2	Labeled MN/ mm^2	%
Upper	1293	0	0	1 da	813	26	3.2	3 da	1140	72	6.3
				2 da	722	139	19.3	6 da	629	39	6.2
Middle	525	0	0	1 da	434	2	0.5	3 da	--	--	---
				2 da	1025	4	0.4	6 da	510	0	0.0
Deep	343	0	0	1 da	585	1	0.2	3 da	--	--	---
				2 da	1711	2	0.1	6 da	425	0	0.0

MCP-1 mRNA in macrophages/fibroblasts (MN)

	Normal skin			Peak lesions (1 & 2 day)				Healing lesions (3 & 6 day)			
Skin depth*	Total MN/ mm^2	Labeled MN/ mm^2	%	Day	Total MN/ mm^2	Labeled MN/ mm^2	%	Day	Total MN/ mm^2	Labeled MN/ mm^2	%
Upper	1079	3	0.3	1 da	647	27	4.2	3 da	792	72	9.1
				2 da	601	23	3.8	6 da	1199	154	12.8
Middle	308	0	0	1 da	367	150	40.9	3 da	546	86	15.8
				2 da	380	116	30.5	6 da	518	10	1.9
Deep	280	0	0	1 da	319	91	28.5	3 da	531	50	9.4
				2 da	362	93	25.7	6 da	379	7	1.8

PMN = polymorphonuclears (granulocytes)

MN = Mononuclear cells (mostly macrophages and fibroblasts)

Representative data from single rabbits containing duplicate lesions of each age.

*These 3 areas were added to give the 3 mm^2 results listed in Table 1.
Note: The granulocytes do not label for MCP-1 mRNA.

TABLE 3

Reproducibility of number of cells labeled for chemokine mRNAs in tissue sections of rabbit skin lesions produced by sulfur mustard.

	Total Cells in a 3 mm² area			Labeled Cells in a 3 mm² area			Percent Labeled		
	2 hr	1 day	2 days	2 hr	1 day	2 days	2 hr	1 day	2 days
	670	660	780	0	0	0	0	0	0
NAP-1	460	270	1760	3	0	61	0.7	0	3.5
in	390	620	400	0	3	10	0	0	2.5
PMN	110	400	2100	0	1.4	91	0	0.3	4.3
Mean ±S.E.M.	408 ±116	488 ±92	1260 ±401	0.8 ±0.8	1.1 ± 0.7	41 ±22	0.2 ±0.2	0.1 ±0.1	2.6 ±1.0
	1730	1880	2490	0	17	172	0	0.9	6.9
NAP-1	1760	2500	4060	31	57	217	1.8	2.3	5.3
in	2250	2710	2010	18	135	70	0.8	5.0	3.5
MN*	2190	1800	3310	76	29	145	3.5	1.6	4.4
Mean ±S.E.M.	1983 ±138	2223 ±225	2968 ±452	31 ± 16	60 ± 27	151 ±31	1.5 ± 0.8	2.5 ± 0.9	5.0 ± 0.8
		2 days	3 days		2 days	3 days		2 days	3 days
MCP-1		1110	1660		230	210		17.3	11.1
in		1520	5550		200	680		12.9	12.3
MN*		3080	5100		460	150		15.0	2.9
Mean ±S.E.M.		1903 ±600	4103 ±1229		297 ±82	347 ±168		15 ±1.0	8.8 ±3.0

*MN = mononuclear cells (mostly macrophages and fibroblasts).
S.E.M. = standard error of the mean

Figure 1. NAP-1 (IL-8) mRNA in a rabbit 3-day dermal sulfur mustard (SM) lesion. Granulocytes (accumulating under the dead epithelium) stain positively for NAP-1 mRNA. This mRNA probably produces NAP-1 protein, which attracts still more granulocytes to the area. The crust (or scab), which contains numerous dead granulocytes is highly effective in keeping the lesion free of infection. Depicted is a frozen section of cold-paraformaldehyde-fixed SM lesion, hybridized with ^{35}S-labeled antisense NAP-1 RNA, autoradiographed, and counterstained with Giemsa. The control ^{35}S-sense NAP-1 RNA probe did not label any cell. X 500.

Glossy prints will be provided after approval of this report has been obtained.

Figure 2. MCP-1 mRNA in a rabbit 1-day dermal sulfur mustard (SM) lesion. SM directly or indirectly (via primary cytokines) caused cells in the macrophage/fibroblast group to produce MCP-1 mRNA. Four such cells, labeled with the MCP-1 RNA probe, are shown. In normal skin, very few cells were labeled. Depicted is a frozen section of cold-paraformaldehyde-fixed SM lesion, hybridized with ^{35}S-labeled antisense MCP-1 RNA, autoradiographed, and counterstained with Giemsa. The control ^{35}S-sense MCP-1 RNA probe did not label any cell. X 500.

Glossy prints will be provided after
approval of this report has been obtained.

Figure 3. GRO mRNA in two hair follicles (one with an attached sebacious gland) from the skin of a rabbit topically exposed in vivo to sulfur mustard (SM) 2 hr previously. Several hair follicle epithelial cells are labeled with the antisense ^{35}S-RNA probe. In normal skin, very few hair follicle cells were labeled. The presence of many hair follicle keratinocytes containing GRO mRNA suggests that, in rabbits, GRO causes keratinocyte proliferation. These cells then migrate out of the follicle and replace the epidermal cells killed by the SM. Depicted is a frozen section of cold-paraformaldehyde-fixed SM lesion, hybridized with ^{35}S-labeled antisense GRO RNA, autoradiographed, and counter-stained with Giemsa. The control ^{35}S-sense GRO RNA probe did not label any cell. X 450.

Glossy prints will be provided after approval of this report has been obtained.

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Figure 4. An overview of the roles of cytokines and other inflammatory mediators produced in skin by irritants such as sulfur mustard, adapted from Kupper (10). Irritants apparently have a direct effect on the keratinocytes of the epidermis and hair follicles, as well as on mast cells and nerves. They may also irritate vascular endothelial cells and local fibroblasts and histiocytes. All of these stimulated cells (including the mast cells) would then release primary cytokines, such as IL-1 (beta) and TNF (alpha)).

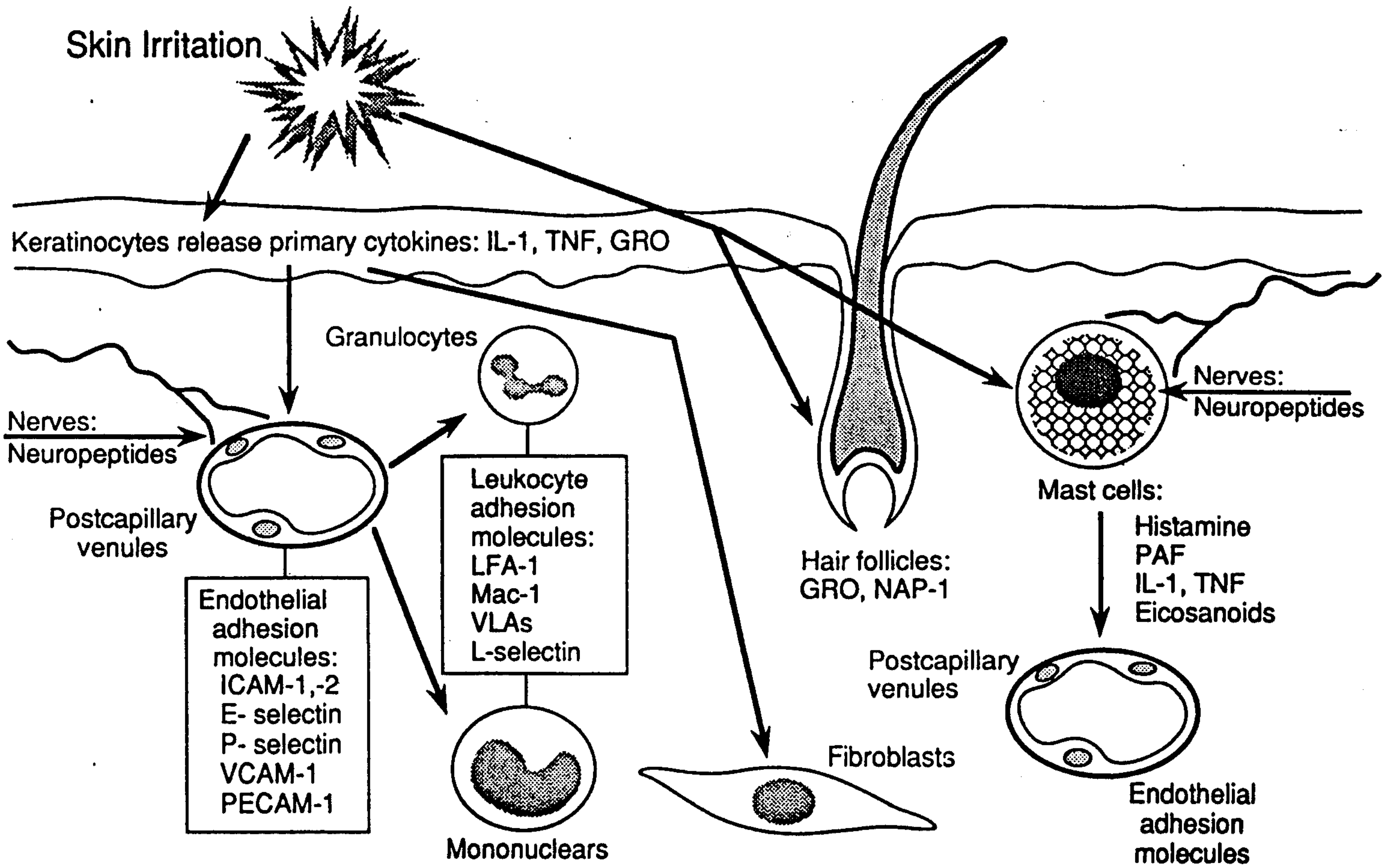
The primary cytokines stimulate the production of adhesion molecules, both in vascular endothelial cells* and in local intravascular leukocytes (56). The leukocytes then adhere to the endothelium and migrate into the tissues. In almost every cell-type present (including the infiltrating cells which are now plentiful), the primary cytokines also stimulate the production of additional primary cytokines, as well as several secondary cytokines, such as NAP-1 (IL-8), MCP-1, TGF (beta), and GM-CSF. Receptors for cytokines and adhesion molecules are also up-regulated. Since many of these cytokines are chemotactic, they are a major cause of the cell infiltration in inflammatory lesions.

Mast cells are specialized cells that play a major role in both the early and later stages of the inflammatory response. They are extremely sensitive to all types of skin irritation, releasing histamine and eicosanoids, as well as cytokines (see text). Mast cells, therefore, seem to cause the initial vascular response, and then participate with the other cells in producing cytokines that maintain this response.

Our studies suggest that GRO is a primary cytokine of epithelial cells, and that it plays a major role in re-epithelialization (from hair follicles in the rabbit).

* **Note:** With immunohistochemical techniques, we found that SM caused vascular endothelium to produce the adhesion molecules VCAM and ELAM. However, our data is unsufficient to report at this time.

Figure 4


Skin Irritation
Keratinocytes release primary cytokines: IL-1, TNF, GRO
Granulocytes
Nerves:
Neuropeptides
Postcapillary
venules
Leukocyte
adhesion
molecules:
LFA-1
Mac-1
VLAs
L-selectin
Endothelial
adhesion
molecules:
ICAM-1,-2
E- selectin
P- selectin
VCAM-1
PECAM-1
Mononuclears
Fibroblasts
Hair follicles:
GRO, NAP-1
Nerves:
Neuropeptides
Mast cells:
Histamine
PAF
IL-1, TNF
Eicosanoids
Postcapillary
venules
Endothelial
adhesion
molecules

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Chapter 2

CYTOKINES IN SM LESIONS: OTHER EXPERIMENTS AND COMMENTS

A. In situ hybridization in tissue sections of SM lesions

Unfortunately, in situ hybridization is not an easy technique and a major part of the first year of our contract was spent getting it to work. We used the rabbit model because all of our studies during the past 12 years (see Publication List on pp. 9 to 11 in the front section of this report) were carried out in this species. We tried cDNA and antisense RNA probes from other species (human and mouse), but only rabbit antisense RNA probes worked well in tissue sections of rabbit lesions.

Fixatives: We tried various fixatives to improve the quality of our tissue sections, but none were better than the lightly fixed frozen sections described in the following chapter. With our NAP-1 antisense RNA probe, SM lesions fixed in No-Tox (Earth Safe Industries, Belmead, NJ), Histochoice (Ameresco Co., Solon, OH), and 100% acetone gave poorer in situ hybridizations than those fixed in our standard buffered paraformaldehye. STF (Streck Laboratories, Omaha, NE), however, was just as satisfactory as our standard method.

Embedding. Glycol methacrylate-embedding enables tissue sections to be cut that are far superior to those embedded in paraffin, as well as those that are frozen and cut in the cryostat. Unfortunately, none of the in situ hybridization or immunohistochemical techniques described in this Final Report worked with tissue sections prepared by these other methods.

Transforming growth factor (TGF). Plasmids containing murine transforming growth factors B_1 and B_2 cDNAs were obtained from Genentech, Inc., South San Francisco, CA. We made the ^{35}S-antisense RNA probes from them, but they did not hybridize with the mRNA in any cell present in our SM lesions. (Murine TGFs have a 98% homology with rabbit and human TGFs and therefore riboprobes produced from them should have hybridized well.)

Nonspecific binding of ^{35}S-labeled RNA probes. Another problem was hybridization of our sense RNA probes with the eosinophils in our tissue sections. Such sense probes have the complementary nucleotide sequence of our antisense probes, and therefore are a near perfect "negative" control. Eosinophils contain large amounts of cationic protein. Such positively charged protein would be expected to bind non-specifically to all RNAs (which are negatively charged). This problem was most frequently encountered with our GRO mRNA probes, but GRO antisense mRNA preferentially labeled epidermal and hair follicle cells and

macrophages --- all of which did not label with sense RNA (because they were not rich in cationic protein).

SM-exposed human skin explants and human cytokine probes for mRNA. Finally, we ran a few experiments on human skin. Antisense ^{35}S-RNA probes were made from plasmids containing human cDNA for IL-1 alpha, IL-1 beta, IL-6, IL-8, TNF alpha, IFN gamma, and TGF beta (obtained from Dr. Jeffrey D. Hasday of the University of Maryland School of Medicine, Baltimore, MD.). Discarded 1.0-cm^2 squares of human skin (from mastectomies) were topically exposed to 1% SM and organ-cultured for 3 hours (Nakamura, 1990 - see p. 9 in Publication List in the front section of this report). Tissue sections were made from these skin explants and in situ hybridized with the human probes just listed. A few cells in the dermis of these specimens hybridized with the antisense RNA probes for (human) IL-1 alpha, IL-1 beta and IFN gamma. However, no such hybridization occurred in epidermal cells or with any of the other cytokine probes that were evaluted. We did not pursue these "human-to-human" hybridizations any further because such human specimens contained no infiltration of inflammatory cells.

Comment. Our inability to label the mRNAs of certain cytokines in tissue sections of SM lesions may be due to several factors. (a) The cytokine mRNA evaluated may not have been present in the specimens studied. (b) Certain cytokine mRNAs may be in low abundance, or more readily destroyed by tissue RNases (before our fixatives inactivated them). And, (c) the methodology we are using may work only with some mRNAs and not with others. We often used, as a "positive" control, sections of dermal granulomas produced rabbits by BCG vaccine. Such granulomas contain numerous highly activated macrophages that are known to produce many cytokines. We found, however, that cells in sections of BCG rarely, if ever, were labeled by our probes when cells in sections of our SM lesions were not labeled. In other words, although BCG lesions usually showed more labeling, and labeling of greater intensity, at least a few cells in SM lesions were labeled with the probes that hybridized.

B. Attempts to identify of cytokine proteins in tissue sections of SM lesions

Introduction. In normal human skin, IL-1 alpha protein has been reported to be stored in the epidermis and supposedly released following epidermal injury, thereby initiating the inflammatory cascade of cytokines and other mediators. [IL-1 alpha mRNA is low or absent in normal epidermis.] We wanted to confirm these findings and show that sulfur mustard released IL-1 alpha. This primary cytokine would induce keratinocytes, macrophages, granulocytes, and/or fibroblasts to produce the chemotactic cytokines NAP-1 and MCP-1, the mRNAs of which we have already identified in SM lesions.

Methods. We performed (without success) 17 separate experiments attempting to identify the protein of IL-1 alpha and IL-1 beta in tissue sections by standard immunohistochemical techniques. These experiments involved goat primary antibodies (as well as rabbit primary antibodies) against human IL-1 alpha and human IL-1 beta, as well as goat primary antibodies against rabbit IL-1 alpha and rabbit IL-1 beta. [All of these antibodies were IgG fractions.] With the human specimens, absorption of the primary antibody with purified human keratin (from Sigma Chemical Co.) was tried in six experiments (without success) to stop the non-specific staining of surface keratinocytes by both primary antibodies and the non-antibody IgG controls. Monoclonal mouse antibodies to rabbit IL-1 alpha and IL-1 beta were also tried without success. They were purchased from Cytokine Sciences, Inc., Boston, MA, and tested with the avidin-biotin complex (ABC) kit purchased from Vector Laboratories.

The human skin specimens were discards from surgical operations. They were cut into 1.0-cm^2 pieces, exposed to SM in vitro and organ-cultured, usually for 3 and 16 hours, along with diluent-exposed controls. The culture fluids were cleared by centrifugation and then frozen. Tissue sections were made, and immunohistochemical procedures were performed with rabbit (or goat) anti-human IL-1 alpha and IL-1 beta primary antibody (IgG fraction), biotin-labeled anti-rabbit (or anti-goat) IgG secondary antibody; peroxidase-labeled avidin-biotin complex; and the H_2O_2--diaminobenzidine (peroxidase) substrate.

Tissue sections of rabbit SM lesions, two or three days of age, were also made and immunohistochemical procedures were performed with goat anti-human IL-1 alpha and IL-1 beta primary antibody (IgG fraction), biotin-labeled rabbit anti-goat IgG secondary antibody, peroxidase-labeled avidin-biotin complex, and the H_2O_2--diaminobenzidine (peroxidase) substrate.

Several of these experiments were repeated with the same primary antibodies but with gold-labeled secondary IgG antibodies, followed by silver intensification. Sections of rabbit BCG lesions were sometimes used as "positive" controls to work out the methodology. Gold-labeling eliminates the need to inactivate normal tissue peroxidases prior to performing the immunohistochemical procedures.

Results: Unfortunately, we did not find IL-1 alpha or IL-1 beta in cold formalin-fixed or unfixed cryostat-cut frozen sections in any of these experiments. Our controls of non-antibody IgG stained surface keratinocytes, as well as some of the cells in the tissue sections, with the same intensity as the specific antibodies. Surface keratinocytes still stained non-specifically, in spite of absorption of the primary antibody with purified human keratin, although the staining was reduced. Various antibody dilutions were tried without success. Rabbit broncho-alveolar lavage macrophages are known to be highly activated cells, but even they stained with the same intensity with IgG specific for IL-1

as with control IgG. We are forced to conclude that IL-1 proteins cannot be detected histochemically in SM lesions of rabbits, nor in human skin that was exposed in vitro to SM.

C. Human IL-1 alpha and IL-1 beta released in organ culture.

To prove that IL-1 (released from injured epidermis) triggered the resulting inflammatory response, we assayed culture fluids from the human skin exposed to SM in vitro for IL-1 alpha and IL-1 beta. The ELISA test made by R and D Systems, Minneapolis, MN, was used according to the directions supplied with the kit. This kit provided the appropriate standards, which checked out well. Three-hour culture fluids from SM lesions contained only slightly more IL-1 alpha than those from the methylene chloride controls, but they contained no IL-1 beta. This experiment confirmed the literature that stated that IL-1 alpha is released from injured epidermis. The injury seems to be caused by removing the surgical specimen from its blood supply, as well as by the SM treatment. Such injury apparently released IL-1 alpha into the culture fluids in both our controls and SM treated specimens. [These assays were performed by Paul J. Converse, Ph.D., Assistant Professor, Department of Molecular Microbiology and Immunology, Johns Hopkins School of Hygiene & Public Health.]

Chapter 3

Histochemical Demonstration of Hydrogen Peroxide Production by Leukocytes in Fixed-Frozen Tissue Sections of Inflammatory Lesions

SUMMARY

The production of H_2O_2 by cells in cold paraformaldehyde-fixed frozen sections of inflammatory lesions was histochemically demonstrated by incubating them with diaminobenzidine (DAB) for 2 to 6 hours. Catalase (150 ug/ml, about 1400 units per ml) inhibited the reaction, indicating that H_2O_2 was required to produce the chromogenic DAB product. PMN and eosinophils were the main types of cells stained by the DAB reaction. Positive staining of macrophages was less frequent. The H_2O_2 was produced by metabolic enzymes that were still active after cell death and mild fixation. An atmosphere of 95 to 100% oxygen enhanced the specific DAB reaction, and an atmosphere of 100% nitrogen eliminated it. The DAB histochemical reaction to detect H_2O_2 requires the presence of peroxidases to produce the colored reaction product. Within our tissue sections, such peroxidases were evidently present in excess, because the addition of low concentrations of H_2O_2 significantly increased the reaction product. Although some of the H_2O_2 produced by the granulocytes may have been derived from the dismutation of superoxide (O_2^-), the NADPH-oxidase pathway for O_2^- formation did not seem to be involved: NADPH-oxidase, a rather labile enzyme, should not be active after mild fixation, and diphenyleneiodonium (DPI) (100 uM), an inhibitor of flavine-requiring NADPH-oxidase, did not inhibit the reaction. Reactive nitrogen intermediates were also not involved, because N^G-monomethyl-L-arginine and N^G-nitro-L-arginine methyl ester, inhibitors of nitric oxide synthetase, did not appreciably inhibit the reaction. We conclude that stable, non-flavine-requiring oxidases, possibly cyclooxygenases or lipoxygenases, produced the H_2O_2 measured histochemically by our DAB reaction. These studies were made on tissue sections of acute dermal inflammatory lesions produced in rabbits by the topical application of 1% sulfur mustard (bis(2-chloroethyl) sulfide) (SM) in methylene chloride. Both <u>intact</u> PMN and <u>disintegrating</u> PMN in the base of the crust produced H_2O_2. Despite the production of H_2O_2 and the presence of peroxidase activity, no tissue damage was seen microscopically near the H_2O_2-producing cells, which indicates that the tissues are well protected by the antioxidants present in this self-limiting inflammatory reaction.

ABBREVIATIONS:

ATZ	-	3-amino-1,2,4-triazole
BCNU	-	1,2-bis-[2-chloroethyl]-1-nitrosourea
DAB	-	3,3'-diaminobenzidine tetrahydrochloride
DDTC	-	diethyldithiocarbamate
DPI	-	diphenyleneiodonium
FAD	-	flavine adenine dinucleotide
GM-CSF	-	granulocyte-monocyte colony stimulating factor (a cytokine)
GSH	-	glutathione
HEPES	-	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] was the buffer used in these experiments
H_4B	-	5,6,7,8-tetrahydrobiopterin
IL-4	-	interleukin 4
NADPH	-	B-nicotinamide adenine dinucleotide phosphate, reduced form
NAME	-	N^G-nitro-L-arginine methyl ester
NMMA	-	N^G-monomethyl-L-arginine
PEC	-	peritoneal exudate cells
RNIs	-	reactive nitrogen intermediates
ROIs	-	reactive oxygen intermediates
SM	-	sulfur mustard (bis(2-chloroethyl) sulfide)
SOD	-	superoxide dismutase
TNF	-	tumor necrosis factor (a cytokine)

KEY WORDS: Diaminobenzidine; Catalase; Superoxide, Nitric oxide; Sulfur mustard; Granulocytes (PMN); Macrophages.

INTRODUCTION

Reactive oxygen intermediates (ROIs) are produced by the phagocytes that infiltrate inflammatory lesions. Although ROIs help the host destroy invading microorganisms (1,2), they may also damage host tissues (3-9).

Briggs and Karnovsky (10-13) pioneered the histochemical demonstration of the ROIs, hydrogen peroxide and superoxide (O_2^-), mainly using isolated granulocytes and electron microscopy. Similar methods were also used for light microscopy (14-18). We modified these methods by using diaminobenzidine (DAB) with longer incubation times, and then applied the technique to study the production of H_2O_2 within frozen tissue sections from fixed dermal inflammatory lesions produced in the skin of rabbits by the military vesicant, sulfur mustard (SM). We found that oxygen was metabolized to H_2O_2 by still-active non-flavine enzymes of intact, as well as disintegrating, granulocytes, and that the H_2O_2 produced caused no apparent tissue damage.

MATERIALS AND METHODS

Sulfur mustard (bis(2-chloroethyl) sulfide) (8 ul of a 1.0% solution in methylene chloride) was topically applied at various times to multiple sites on the flanks of rabbits, so that, by the time the animals were sacrificed, 1-, 2-, 3- and 6-day SM lesions were present (19,20).

Central 3- to 4-mm sections of these lesions were mechanically shaken for 4 hr in cold (4°C) 4% paraformaldehyde fixative prepared in 0.1 M sodium phosphate buffer (pH 7.2 to 7.4). Then, they were shaken overnight in cold (4°C) 20% sucrose in phosphate-buffered saline (PBS) (containing 0.01 M sodium phosphate (pH 7.2 to 7.4) and 0.15 M NaCl). The next morning, they were shaken for 2 hr in cold (4°C) 5.0% glycerol--20% sucrose in PBS, and were then embedded in OCT compound (Lab-Tek Division, Miles Laboratories, Inc., Naperville IL) in plastic molds (Cryomolds, Lab-Tek), "snap" frozen in liquid nitrogen, wrapped in Parafilm (American National Can Co., purchased from Curtin Matheson Scientific, Inc., Jessup, MD), and stored in an airtight plastic container at -80°C until used. Frozen sections were cut in a cryostat at 4 to 6 um, put onto precleaned silane-coated slides (Superfrost Plus, Fisher Scientific Co., Pittsburgh, PA), and air-dried with a cool hair drier. They were used either on the same day or on the next day after storage at -80°C in a tape-sealed slide box containing silica gel desiccant. [When we stored the tissue sections, on slides, at -80°C for about three weeks before running the DAB reaction, the reaction product was only slightly less intense than the reaction product found in tissue sections incubated with DAB within 24 hrs.]

The tissue sections were incubated for 2 to 6 hrs at 37°C at pH 6.7 to 7.4 in 0.1 M HEPES buffer, (Sigma Chemical Co., St. Louis, MO, Cat. No. H-3375), containing glucose (1.0 mg/ml), and 3,3'-diaminobenzidine tetrahydrochloride (1.0 mg/ml) (Sigma, Cat. No. D-5637) (Table 1). [Due to the acidic nature of the DAB hydrochloride, the pH of the reaction mixture was 0.1 to 0.2 units lower than that of the HEPES buffer.] Between 10 and 18 hrs, the reaction product and background staining were darker than at 4 to 6 hrs, but, after 10 hrs, there was little or no increase in intensity. Therefore, for evaluating the effects of inhibitors and activators, overnight incubation was not as satisfactory as 4- to 6-hr incubation. When TRIS buffer (0.1 m) (Sigma) was used instead of HEPES buffer, similar results were obtained.

In our early experiments, the slides were subsequently placed in 5% $CoCl_2$ in HEPES buffer for 25 min at 23°C to intensify the reaction product (16,17). Then, they were washed in 0.9% NaCl, counterstained with hematoxylin (Sigma) for 20 min at 23°C, washed in deionized water, dehydrated in 50, 70, 95 and 100% ethanol, dipped in xylene, and covered with a coverslip, using Permount (Fisher Scientific Co.). When quantitation of the intensity of the reaction was required, the $CoCl_2$

intensification and the counterstain were usually omitted. Therefore, most of the results reported in Tables 1 and 2 were from tissue sections that were neither cobalt-treated nor counterstained. $CoCl_2$ intensification, however, facilitates the identification of H_2O_2-producing cells in counterstained preparations.

We included catalase (150 ug/ml, about 1400 units per ml) (Sigma, Cat. No. C-40) as a control in a duplicate reagent solution. Catalase is the classic enzyme that destroys hydrogen peroxide. Since catalase prevented the formation of the colored DAB reaction product, this reaction is a measure of H_2O_2 formation in the tissue sections. At this concentration of catalase, pre-incubation of the tissue sections at room temperature for 10 to 15 min with catalase alone (before they were placed in the reagent solution) was required to make the destruction of H_2O_2 complete. Pre-incubation was also used with every inhibitor that we investigated.

The data from 3 or 5 investigators were collected and pooled. The investigator reading the slides usually did not know whether enhancement or inhibition was expected, and multiple confirmatory experiments were done to be sure the result was reproducible.

RESULTS AND INTERPRETATION

Production of H_2O_2 in developing and healing sulfur mustard lesions

The amount of H_2O_2 produced, i.e., the amount of DAB oxidized into an insoluble histochemically visible product which was inhibitable by catalase, was proportional to the number of granulocytes (PMN) present. The oxidation of DAB by H_2O_2 is not direct, but is dependent upon the presence of myeloperoxidase which utilizes H_2O_2 as its substrate.

One- to 3-day SM lesions had high numbers of PMN in the dermis (19,20); healing (6-day) lesions had a decreased number of PMN there. The crusts of 3-day and 6-day (healing) lesions contained numerous live and disintegrating PMN (19,20). Most of the intact PMN and many disintegrating PMN produced H_2O_2 (Figure 1). In healing 3-day SM lesions, new epithelium grew unharmed under the crust, which was rich in H_2O_2-producing live and disintegrating PMN (Figure 1).

In tissue sections of SM lesions, some macrophages (and probably some activated (20) fibroblasts) produced H_2O_2, i.e., they seemed to oxidize DAB into an insoluble histochemically visible product, which was inhibitable by catalase. We could not readily differentiate eosinophils from PMN in these frozen sections, as rabbit PMN (called heterophils) contain red-orange granules (21,22). However, in glycol methacrylate-embedded tissue sections of SM lesions (stained with Giemsa), only low percentages of eosinophils were present (19). The few mast cells that we could identify produced little, if any, H_2O_2.

In order to be certain that the rabbit macrophages could produce the DAB reaction product, we collected normal rabbit alveolar macrophages (AM) by broncho-alveolar lavage after the animal was sacrificed. The AM present, both in smears and in fixed-frozen sections of the AM pellet obtained by centrifugation, oxidized DAB, even though AM have different peroxidases than granulocytes have (see 23). However, different AM preparations gave widely differing results: In some, only a few AM were stained, whereas in others, over 90% were stained. Even different parts of the same smear might stain with different intensities. AM are known to contain relatively high concentrations of catalase (24), but we suspect that this variability was due to how well certain metabolic enzymes were preserved in the preparation and, perhaps, how well atmospheric oxygen reached the appropriate sites within the cells. The staining of the intact and disintegrating PMN in tissue sections of SM lesions showed little variability and was much more reproducible than the staining of alveolar macrophages.

Inhibitors and activators of the histochemical reaction

Various inhibitors and activators of H_2O_2 production were tested, in order to determine which oxidants (with tissue peroxidases) produced the visible DAB reaction product. The concentrations of these modulators, and their effects on the histochemical reaction, are listed in Table 2. We have diagrammed the pertinent respiratory pathways in Figure 2, so that the reader can readily understand these effects.

Atmospheric oxygen (95 to 100%) and nitrogen (100%). Oxygen is the ultimate source of ROIs and, therefore, an important substrate for the histochemical reaction (Figure 2). When we carried out the histochemical reaction in 95 to 100% oxygen (instead of air) in a sealed anaerobic-type jar, usually more orange-brown DAB reaction product was produced (Table 2). This reaction was presumably due to H_2O_2 and not due to the direct action of O_2^- on DAB, because catalase (1400 units/ml) almost completely prevented the DAB reaction product from forming.

An anaerobic atmosphere completely stopped the production of oxidized DAB. This anaerobic atmosphere was produced by bubbling N_2 gas into the reagent solution before its application to the slides containing the tissue sections and then incubating the slides in an anaerobic-type jar filled with N_2.

Catalase. Catalase (150 ug/ml, about 1400 units/ml, and above) (Sigma, Catalog No. C-40) prevented the formation of the DAB reaction product (by breaking down H_2O_2 into H_2O and O_2). Therefore, catalase identified H_2O_2 as the main reactive oxygen intermediate detected by our histochemical reaction.

Effect of pH. The intensity of the reaction product was fairly constant over a pH range of 6.7 to 7.4. We did not evaluate higher or lower pHs.

Superoxide (O_2^-). Phagocytes produce H_2O_2 during their respiratory burst (1,2). A large part of H_2O_2 comes from the dismutation of O_2^- (Figure 2). However, when exogenous superoxide dismutase (SOD) (3200 units/ml) (Sigma, Cat. No. S-2515) was added to the DAB reaction mixture, no appreciable effect was found. SOD converts superoxide (O_2^-) into H_2O_2 and O_2. Thus, in our tissue sections, O_2^- did not directly oxidize DAB into the orange-brown insoluble reaction product. This conclusion is also supported by the absence of the DAB reaction product in the presence of catalase (see above), which breaks down H_2O_2, but not O_2^-.

Diethyldithiocarbamate (DDTC) (10 mM), inhibited the DAB reaction almost completely. DDTC is a thiol-delivery agent (reducing H_2O_2) and a free-radical scavenger, as well as a metal chelator and an SOD inhibitor (reviewed in 25-27). Thus, there are many reasons why DDTC could inhibit this histochemical reaction.

Flavine adenine dinucleotide (FAD) (0.6 mM), is a cofactor for the production of superoxide by NADPH oxidase (2). FAD often increased the amount of DAB reaction product, both in air and in 95% O_2 (Table 2). Catalase (1400 units/ml) almost completely inhibited the DAB reaction when FAD was present. Therefore, if more O_2^- was formed when the cofactor FAD was added, it was probably dismutated to H_2O_2.

Diphenyleneiodonium (DPI), an inhibitor of all nucleotide-requiring flavoprotein enzymes (28,29), (10 uM and 100 uM) had no effect on the amount of DAB reaction product produced. DPI in these concentrations should have completely inhibited NADPH- oxidase, which is the main source of O_2^- and then (by dismutation) of H_2O_2 in PMN (see Figure 2). These experiments indicate that O_2^- did not directly oxidize the DAB to produce the visible reaction product, and that the H_2O_2 detected histochemically by DAB was produced by oxidases that did not use FAD as a co-factor.

Nitric oxide (NO). NO reacts with O_2^- to form peroxynitrite, which is a strong oxidant. NO is produced by macrophages (30-32), PMN (32,33) and other cells (31,32,34,35). Therefore, NO, in addition to H_2O_2, might produce a DAB reaction product in our tissue sections (with the appropriate tissue enzymes). Catalase is known to cause oxidation (and therefore inactivation) of tetrahydrobiopterin (H_4B) (36), a co-factor required for NO synthesis from L-arginine (37-39). Therefore, the prevention of the DAB color reaction by catalase does not rule out the participation of NO (and peroxynitrite).

The local production of NO should be increased by adding arginine, H_4B and/or NADPH (38,39). However, under our experimental conditions, no increased formation of the DAB reaction product was observed (Table 2). Conversely, N^G-

monomethyl-L-arginine (NMMA), and N^G-nitro-L-arginine methyl ester (NAME), known inhibitors of NO synthesis (31), did not decrease the amount of reaction product (Table 2). Thus, H_2O_2, and not nitric oxide, apparently produced the DAB reaction product that we observed in our tissue sections.

Additional proof that NO was not responsible for our DAB reaction comes from one experiment we performed on mouse peritoneal exudate cells (PEC). Mouse PEC were produced and activated <u>in vivo</u> with an intraperitoneal (i.p.) injection of about 40 million live attenuated tubercle bacilli (BCG) followed, after 19 days, by 1 ml of 10% peptone (Difco Laboratories, Detroit, MI) i.p. (40). The PEC were collected 2 days later and incubated in vitro for 24 hr with E. coli lipopolysaccharide, serotype 0128:B12 (Sigma) (20 ng in 1.0 ml RPMI 1640 culture medium) (40).

Coverslips were placed on the bottom of the culture dishes to collect adherent macrophages. These adherent activated macrophages were air dried, stored overnight at -80°C, and then incubated overnight in our standard DAB-glucose reagent. Very few cells showed the colored histochemical reaction product; and, in the presence of 0.15% catalase (10X), no cells were stained. After centrifugation, however, the culture fluids were assayed for nitrites (41,42) and showed the expected increase over 0-hr controls, i.e., about 1.2 ug of sodium nitrite (17 nanomoles) were produced by 1.3 million PEC in 24 hr. Therefore, these mouse PEC were producing NO in culture; and this NO (or the peroxynitrite formed from it) did <u>not</u> produce (with the cellular enzymes present) our DAB reaction product.

In other experiments, <u>rabbit</u> pulmonary alveolar macrophages (AM) were incubated overnight with endotoxin, as described above. No appreciable increase in nitrites was found in the culture fluids; therefore, under these conditions, rabbit AM produced very little NO. Nonetheless, a proportion of these AM (when smeared on glass slides) showed a positive histochemical reaction with DAB. Rabbit AM are known to be poor NO producers (J.B. Hibbs, Jr. and D.L. Granger, personal communication).

<u>Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH)</u>. The reducing co-factors, NADPH (0.64 mM) and glutathione (5.0 mM), also inhibited the DAB reaction to various degrees (Table 2). These reducing cofactors probably destroyed H_2O_2 directly via the glutathione peroxidase in the tissue sections (see Figure 2). Apparently, GSH peroxidase does not react with DAB to produce the visible reaction product. This conclusion was confirmed by the fact that bis-[chloroethyl]-nitrosourea, an inhibitor of GSH reductase (43), had little effect on this histochemical reaction (see Figure 2).

<u>Manganese chloride</u>. $MnCl_2$ (11) has been used to enhance a DAB reaction. In our system, $MnCl_2$ (0.5 mM and 5.0 mM) was only slightly enhancing.

Aminotriazole (ATZ) and sodium azide. ATZ (12,14) and NaN_3 (31,16,17) were used by others to enhance the reaction by inhibiting endogenous catalase. [Endogenous catalase could break down H_2O_2 before it reacted with the histochemical substrate.] However, in our experiments, ATZ (20 mM and 200 mM) and NaN_3 (1 mM and 100 mM) reduced (rather than enhanced) the amount of reaction product (see Table 2), probably because they are also myeloperoxidase inhibitors (2,14).

Hydrogen peroxide and endogenous peroxidases. In histochemical reactions, DAB is not appreciably oxidized, unless peroxidases are present in the tissues (44,45). To identify the presence of such peroxidases, we added H_2O_2 (0.0013% and 0.0003%) to our standard incubating solution: The H_2O_2 intensified the DAB reaction product considerably (Table 2). This finding indicates that tissue peroxidases are present in excess, and that the production of H_2O_2 (not the tissue peroxidase levels) determines the rate of the DAB reaction.

The effect of H_2O_2 depends on its concentration. At the above low concentrations (0.0013% and 0.0003%), H_2O_2 increased the DAB reaction product in granulocytes and erythrocytes. At 0.02% and 0.005% concentrations, H_2O_2 reduced the reaction product in granulocytes, but enhanced the pseudoperoxidase DAB reaction product found in erythrocytes. Excess H_2O_2 is a known inhibitor of endogenous peroxidases (44). In an experiment not listed in the tables, horseradish peroxidase (HPO) (Sigma, Cat. No. P-8375) was included in our standard buffered DAB-glucose histochemical solution in three concentrations: 400, 40 and 4 ug per ml. This enzyme completely inhibited the positive staining found in controls without HPO. These results suggest that the HPO in solution rapidly utilized the H_2O_2 produced by the cells in the tissue sections to oxidize the DAB in solution, and that no H_2O_2 remained locally to stain the cells that produced it.

Heat. We also heated the tissue sections for 5 min in steam at 100°C prior to performing the DAB histochemical experiment. Such heating prevented the reaction with DAB from occurring, probably by destroying the peroxidases, as well as the enzymes that formed H_2O_2. The addition of the exogenous H_2O_2 (in low concentrations) did not restore a positive reaction to heated tissue sections, which indicates that the heating did destroy the endogenous peroxidases.

Pseudoperoxidase. The hemoglobin of erythrocytes caused a positive DAB reaction in our fixed-frozen cryostat tissue sections (see 44 and 46). Evidently, the iron-heme complex within erythrocytes (plus ambient O_2) produced H_2O_2 and catalyzed the oxidation of DAB. Because of the requirement of H_2O_2, this pseudoperoxidase reaction was also inhibited by catalase (0.15 mg/ml, i.e., 1400 units/-ml). (See also discussion in 47.) Oxygen (95%) significantly enhanced the pseudoperoxidase DAB reaction of erythrocytes. In Tables 1 and 2, we only listed the H_2O_2 production by PMN, although in our records we noted the presence or absence of

the pseudoperoxidase activity of erythrocytes. Imidazole (10 mM) somewhat enhanced the DAB reaction produced by PMN but, at this concentration, did not appreciably inhibit the pseudoperoxidase of erythrocytes (see 48,49 and 50).

DISCUSSION

Specificity of our histochemical reaction for H_2O_2

Catalase, which destroys H_2O_2 (forming H_2O and O_2), prevented the formation of the orange-brown DAB precipitate produced histochemically in the cells of dermal SM inflammatory lesions (Table 1). Therefore, H_2O_2 was probably the main reactive oxygen intermediate (ROI) that, with a peroxidase, oxidized the diaminobenzidine (DAB) substrate to produce the reaction product. The peroxidases, required for the DAB histochemical reaction to take place (44), were evidently present in the tissue sections because the addition of exogenous H_2O_2 (in low concentrations) produced an increase in the amount of the DAB reaction product (Table 2). Since PMN were the main positive-reacting cells seen in the sulfur mustard (SM) lesions, PMN myeloperoxidase was probably the major peroxidase involved.

Source of the H_2O_2 produced these tissue sections

Many inhibitors and activators of H_2O_2 production were evaluated in our DAB histochemical reaction (see Results Section and Table 2). Due to the lack of effect of diphenylene iodonium, we concluded that other oxidative enzymes than those requiring flavine were involved (28,29). In other words, the H_2O_2 did not come from the dismutation of superoxide produced by NADPH oxidase (see Figure 2), which is a major metabolic pathway of PMN. NADPH oxidase is known to be a rather labile enzyme, so it was not surprising that no evidence of its activity was found in tissue sections fixed for 4 hr in cold 4% paraformaldehyde. The actual source of the H_2O_2 produced by the granulocytes in our tissue sections remains undetermined. One or more of the stable oxidases (such as the cyclooxygenase and lipoxygenase of the eicosanoid systems) are good candidates.

Other oxidants that might oxidize DAB

Reactive nitrogen intermediates (RNIs) do not seem to be involved in the oxidation of DAB. The most reactive RNI is peroxynitrite, formed from NO and O_2^-. Exogenous superoxide dismutase (SOD), which destroys O_2^-, had no effect on the oxidation of DAB. Monomethylarginine (NMMA) and N^G-nitro-L-arginine methyl ester (NAME), which inhibit the formation of NO, also had no effect. Thus, peroxynitrite could not have produced our colored DAB reaction product.

Singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^\cdot$) are both reactive enough to oxidize DAB, but are in general not generated in sufficient quantities by PMN to form DAB precipitates (51). Also, hypochlorous acid (HOCl) and chloramines could conceivably oxidize DAB (Figure 2). When HOCl was evaluated, it did not do so (51). Chloramines were not evaluated. However, since chloramines (like HOCl)

are formed from H_2O_2 (by a myeloperoxidase-dependent mechanism) (7,51), it matters little whether H_2O_2 or chloramines actually did oxidize the DAB. In either case, granulocytes produce the H_2O_2 and contain the myeloperoxidase. Catalase, by destroying H_2O_2, prevented the formation of our colored DAB reaction product, and azide (100 mM), a known myeloperoxidase inhibitor, markedly reduced it (Table 2).

Non-enzymatic staining by oxidized DAB

Could oxidized DAB be present in the DAB reagent or formed non-enzymatically during the long (5-hr) incubation time (52)? Oxidized DAB could then act as a dye and stain the PMN and erythrocytes nonspecifically (52). This possibility seems unlikely because: (a) catalase prevented the reaction; (b) steam heat (5 min at 100°C) destroyed the reaction; (c) reducing agents (glutathione and diethyldithiocarbamate) eliminated the reaction; (d) high concentrations of H_2O_2 (0.02%) inhibited the reaction, yet more oxidized DAB should be found in the presence of 0.02% H_2O_2; (e) the addition of horseradish peroxidase prevented the reagents from staining the leukocytes; (f) the reaction product was not produced under anaerobic conditions (100% N_2); and (g) positive (++ to ++++) staining of granulocytes (and erythrocytes) occurs with incubation times as short as 1.5 hrs.

Comparison with similar histochemical reactions reported in the literature

In our experiments, in contrast to some of those reported in the literature (12,13,15-17), inhibition of endogenous catalase by aminotriazole or sodium azide was not required for a good positive DAB reaction, nor were additional manganese ions required for this histochemical reaction (11,51) (Table 2). These discrepancies might be explained by the fact that our method was developed for light-microscopy (not electron-microscopy), with rabbit tissue (not rat or human tissue), and that it required a 2 to 6 hr (not 20 min to 2 hr) incubation at 37°C.

Was there tissue damage by H_2O_2-producing cells?

By light microscopy, in glycol methacrylate-embedded tissue sections, no necrosis of cells and collagen fibers was found adjacent to PMN in the tissues and in the crust (19,20). Yet, these same live and disintegrating PMN were producing H_2O_2 (Figure 1). Therefore, the H_2O_2 must be in non-toxic concentrations or it must be rapidly inactivated soon after it is formed.

Cells and extravasated serum can protect tissues from oxidant damage in many ways (3,53,54). Cells contain superoxide dismutases, catalases and peroxidases (Figure 2). Superoxide dismutase also stops the production of peroxynitrite ($ONOO^-$) (from O_2^- and NO) (55). Serum contains antioxidants, such as ceruloplasmin and albumin. The latter is the major antioxidant in extracellular fluids (56). It is sometimes called a sacrificial antioxidant (56), because its oxidation spares more vital host components. Also, tissues contain micro-nutrient antioxidants: tocopherol (vitamin E) (57), ascorbic acid (vitamin C) (57) and

beta-carotene (a precursor of vitamin A) (53). Only when all of these "shields" are inadequate does local damage occur (3,7).

Thus, it was not surprising that we found no evidence of tissue damage in the dermal SM lesions, which contain large amounts of extravasated serum (58,59). Even under the lesion crust, which produces major amounts of H_2O_2, the tissues appear to be viable. In fact, during healing, keratinocytes readily migrate beneath the crust (from the edge of the wound and from the hair follicles surviving in the wound) with no interference from the high concentration of H_2O_2 (Figure 1).

Cell death and the persistence of oxidative enzymes

None of the cells are viable in frozen sections of tissues. The histochemical reaction product seen is produced by oxidative enzymes that are still active after the cell has died. In vivo, enzymes producing H_2O_2 must also be stable for many hours after cell death. Otherwise, the disintegrating cells in the crust would not have stained.

Reactive oxygen intermediates (ROIs) and the inflammatory response

ROIs are an important part of the host's integrated inflammatory response to injury. They are produced by infiltrating PMN, eosinophils and monocytes (2). The production of ROIs by cells is influenced by cytokines (60,61). Tumor necrosis factor (TNF) (alpha and beta) and granulocyte-monocyte colony stimulating factor (GM-CSF) are the major cytokines activating PMN (60). Interleukin 4 (IL-4), from the Th2 subset of activated T cells, down-regulates ROI production by human mononuclear phagocytes (62).

Within a cell's phagosome-lysosome system, ROIs, hydrolytic enzymes and iron-binding substances (e.g., lactoferrin) work in synchrony (7). For example, the ROIs activate procollagenase (7,63) and inactivate $_1$-proteinase inhibitor (7,64). In this case, the ROIs would enhance proteolytic activity. Lysosomal components may also be secreted, or regurgitated, from the cell (65) or released when the cell dies. However, damage to host tissues by these lysosomal components occurs only when there is a local derangement of host control systems (reviewed in 3 and 7). Evidently, such derangement did not occur in the uncomplicated, slowly developing chemical burn produced by the topical application of dilute sulfur mustard. In other words, damage to tissues by leukocyte oxidants apparently does not occur in all inflammatory reactions.

Acknowledgments

Brian H. Schofield, Jay B. Rao, Theresa T. Dinh, Ki Lee, Marc Boulay, and Drs. Yasuharu Abe, Junji Tsuruta, and Marla J. Steinbeck were co-investigators in these studies. Dr. Steinbeck is in the Department of Pathology, Harvard Medical School, Boston, MA.

We are grateful to Jane Hong, Mike Lee, Richard Bang, and Rena Ashworth for performing some of the experiments described here, and to Ilse M. Harrop for her editorial help with the manuscript. We are also grateful to Drs. Morris J. Karnovsky, Harvard Medical School, and John B. Hibbs, V.A. Medical Center, University of Utah, Salt Lake City, for their critical help in interpreting our results and for suggesting the use of diphenyleneiodonium. In addition, Drs. Andrew R. Cross, Scripps Research Institute, La Jolla, CA, and Michael A. Trush, of our Department, made valuable suggestions concerning these studies. Dr. Cross also provided the diphenyleneiodonium that we used.

Table 1

Effect of various procedures on our standard DAB histochemical reaction

Procedures	Results	Number of times performed
Standard procedure[a] (4- to 6-hr incubation)	++++	10
Unfixed frozen sections	++	7
No glucose	+++ to ++++	5
Post-incubation in $CoCl_2$ (5%)[a]	+++++	10
Standard procedure[a], but incubated overnight, 16 hr	++++++	10
Heat: Steam, 5 min, 100°C	0	3
Same plus H_2O_2 (0.0013% and 0.0003%)	0	3
6-day SM lesions	+++ to ++++	7
Aldehyde fixation[b]	0	4
Aldehyde fixation[b] with the addition of 0.02% H_2O_2 to our standard procedure[c]	++	3

Table 1 Footnotes

a For our standard procedure, 3-day SM lesions were fixed for 4 hr at 4°C in buffered 4% paraformaldehyde. They were frozen in liquid nitrogen and sectioned at 8 um in a cryostat. The tissue sections were stored at -80°C for 1 to 3 days and were then incubated with glucose (1.0 mg/ml) and diaminobenzidine (1.0 mg/ml), at 37° from 4 to 6 hr. In some of the experiments reported in this Table and Table 2, $CoCl_2$ was used (after the reaction occurred) to intensify the colored product. If so, allowance was made for the cobalt intensification in reporting the results in this table.

b Buffered formaldehyde (3.3%) and glutaraldehyde (5%) for 18 hr at 23°C.

c This experiment suggests that the tissue enzymes that produce H_2O_2 are inactivated more readily by aldehyde fixation than are the peroxidases that catalyze the H_2O_2-DAB reaction.

Table 2

Effects of activators and inhibitors on the DAB histochemical reaction[a]

Procedures	Final concen-trations	Results	Number of times performed
Standard procedure[a] (4- to 6-hr incubation)	-----	++++	10
95%[b] or 100% O_2	-----	++++ to +++++	7
100% N_2	-----	0	3
Catalase	1400 u/ml (0.015%, 0.15mg/ml)	0 to ±	20
Standard procedure[a] plus catalase, but incubated 16 hr	1400 u/ml (0.15 mg/ml)	0 to ±	7
Superoxide dismutase (SOD)	3200 u/ml	++++	7
Diethyldithiocarbamate (DDTC)	100 mM 10 mM 1 mM	0 0 to ± ± to ++	2 13 6
Flavine adenine (in air) dinucleotide (FAD)[b] (in 95% O_2)	0.6 mM 0.6 mM	++++ to +++++ +++++ to ++++++	4 3
Diphenyleneiodonium (DPI)[c]	100 uM 10 uM	++++ ++++	4 4
L-arginine	0.6 mM 0.06 mM	++++ ++++	2 2
Tetrahydrobiopterin (H_4B)[d]	50 uM	+++ to +++++	1
Nicotinamide adenine dinucleotide phosphate, (reduced form) (NADPH)	0.64 mM 0.32 mM	++ ++ to +++	11 5
Glutathione (GSH)	5 mM	0 to ±	5
N^G-monomethyl-L-arginine (NMMA)	50 uM 5 uM	++++ to +++++ ++++ to +++++	2 1
N^G-nitro-L-arginine methyl ester (NAME)	10 mM 1 mM	++++ ++++	4 4

continued

Table 2 (continued)

Procedures	Final concen-trations	Results	Number of times performed
1,2-bis-[2-chloroethyl]-1-nitroso-urea (BCNU)[e]	100 ug/ml	+++ to ++++	2
$MnCl_2$	5.0 mM 0.5 mM	++++ to +++++ ++++ to +++++	2 3
3-amino-1,2,4-triazole (ATZ)	200 mM 20 mM 2 mM	++ to +++ +++ to ++++ ++++	3 3 3
Sodium azide (NaN_3)	100 mM 1 mM	± to + +++ to ++++	3 3
Additional H_2O_2	0.02% 0.005% 0.0013% 0.0003%	± ++ to +++ +++++ to ++++++ +++++	7 2 3 5

Table 2 Footnotes

a In a given experiment, the results of our standard procedure were always called ++++, and every other procedure in that experiment was compared to that standard.

b 95% O_2 in 5% CO_2; catalase (1400 u/ml) inhibited the reaction almost completely.

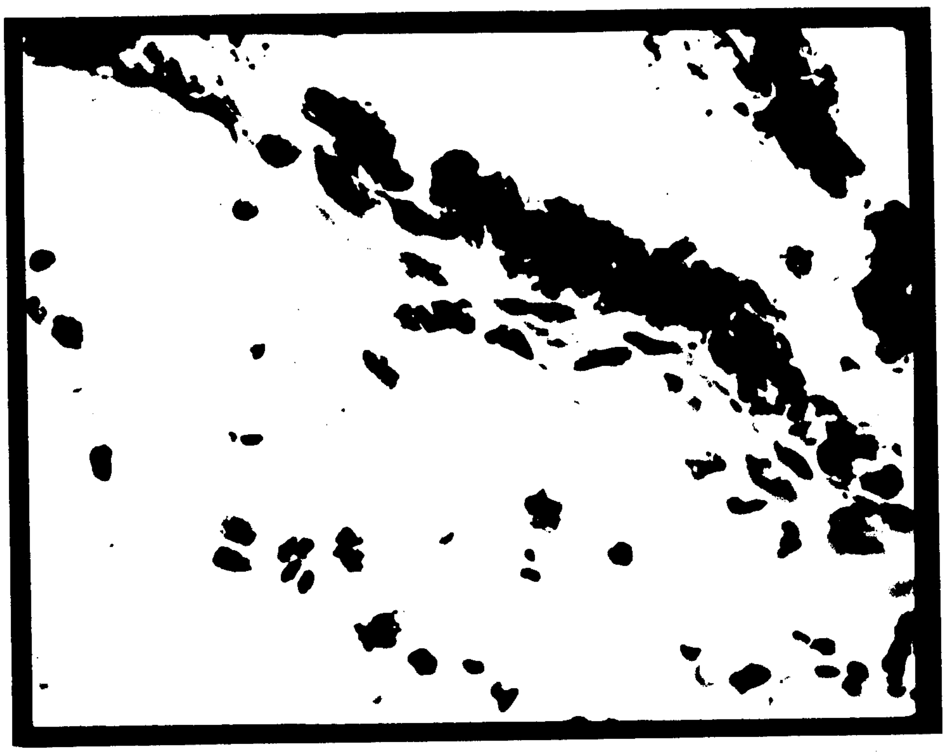
c Supplied by Dr. Andrew R. Cross, Scripps Research Institute, La Jolla, CA 92037

d H_4B plus magnesium acetate (1 mM) plus L-arginine (2 mM) gave similar results. Catalase inhibited this reaction. H_4B was purchased from Dr. B. Schircks Laboratories, Buechstrasse 17a, CH-8645 Jona, Switzerland.

e The bis-[chloroethyl]-nitrosourea (BCNU) (NSC-409962) was supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892 (Dr. V.L. Narayanan and Ms Nancita R. Lomax).

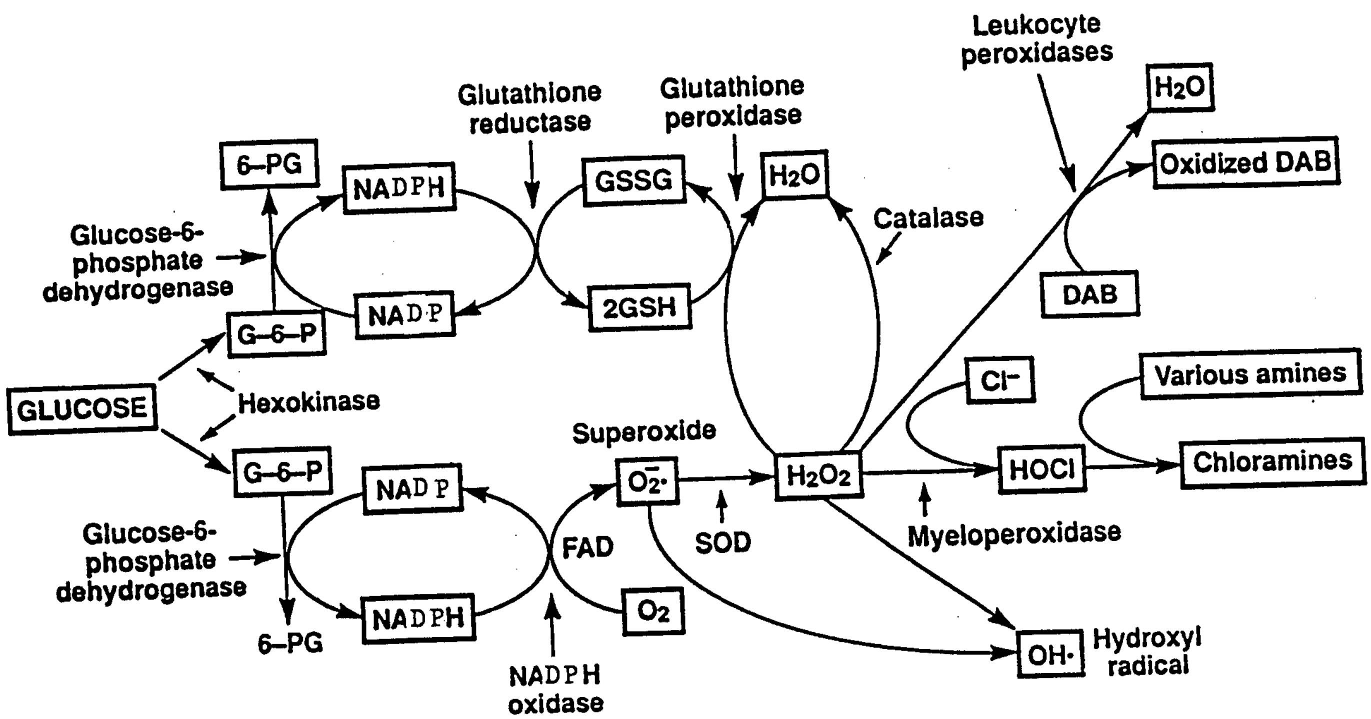
Note: All chemicals used in the experiments herein described were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated.

Figure 1. A healing 3-day (rabbit) SM skin lesion, showing new epithelium growing unharmed beneath live and disintegrating granulocytes in the crust that are actively producing H_2O_2, (shown by the orange-brown reaction product). Thus, these new epithelial cells were apparently totally resistant to any toxic effects that H_2O_2 might have. The fixed-frozen tissue section was incubated at 37°C for 5 hr in diaminobenzidine, glucose, and HEPES buffer (pH 6.8), and then counterstained with Giemsa. X 625.



Glossy prints will be provided after approval of this report has been obtained.

Figure 2. Enzymes and co-factors influencing the production and destruction of hydrogen peroxide. Leukocyte peroxidases plus H_2O_2 oxidize diaminobenzidine (DAB) to the orange-brown insoluble polymeric reaction product that we observe in tissue sections. FAD -- flavine adenine dinucleotide. SOD -- superoxide dismutase. (This figure was derived from several textbooks of biochemistry.)



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Chapter 4

EFFECTS OF NEW INFLAMMATORY INHIBITORS ON SULFUR MUSTARD LESIONS

No specific treatment exists for dermal lesions produced by SM. We therefore obtained and tested a variety of promising new anti-inflammatory agents from several pharmaceutical companies. Most of these agents were injected directly into the SM lesions, beginning 2 hours after the topical application of 1% SM in $MeCl_2$. Some of them were applied topically in a bland ointment base. Both the intra-lesion and topical applications were given twice daily. The following is a list of inhibitors we tested.

3-isobutyl-1-methyl xanthine..Sigma
an inhibitor of cyclic-AMP phosphodiesterase

HWA 486 (Leflunomide). . . . .Hoechst AG
an isoxazol derivative that inhibits macrophage (and lymphocyte) proliferation

A77-1726B. . . . . .Hoechst AG
the active Leflunomide metabolite

NPC 15669. . . . . .Scios Nova, Inc.
a leukocyte recruitment inhibitor (an active leumedin)

NPC 14692. . . . . .Scios Nova, Inc.
a negative "leumedin" control

ETH 615-139. . . . . .Leo, Inc. (in Denmark)
a potent inhibitor of leukotriene synthesis effective in ointments applied to the skin; it also inhibits IL-8 gene expression.

WAY-121,520. . . . . Wyeth-Ayerst
a phospholipase A_2-inhibitor and a lipoxygenase inhibitor (of leukotriene synthesis)

A-64077 Zileuton . . . . . . Abbott
a 5-lipoxygenase inhibitor (of leukotriene synthesis)

L-663,535. . . . . Merck-Frosst
a leukotriene inhibitor

L-656,224. . . . . Merck-Frosst
a leukotriene inhibitor

IL-1ra Interleukin-1 receptor antagonist . . . Synergen, Inc., Boulder, CO/
an inhibitor of IL-1 alpha and IL-1 beta.

Soluble IL-1 receptor (sIL-1R) . . Immunex, Inc. in Seattle, WA.

Soluble TNF receptor (sTNFR:FC). . Immunex, Inc. in Seattle, WA.

Soluble complement receptor 1 (SCR1) . . T Cell Sciences, Cambridge, MA.
an inhibitor of complement (J. Immunol. 146: 250, 1991)

Unfortunately, none of these inhibitors had any pronounced effect on the development or healing of the SM lesions (Tables 1 to 4). This was especially disappointing because the cytokine inhibitors, IL-1ra, sIL-1R and sTNFR:FC, and the complement inhibitor, SCR1, should have had an effect. Perhaps, the dosage was wrong, or the SM rabbit model is different from the other animal models tested by each industrial company. Alternatively, the inhibition of one cytokine may be compensated for by over-production of others.

Tables 1 through 6 follow.

Table #1: Effects of Inhibitors Applied Topically to Dermal Sulfur Mustard Lesions for 4 Days (Rabbit #1)

Inhibitor Name	Company	Description	[ ] in mg/ml	Dosage (mg)	Solvent	Lesion Size x (Thickness) in mm	Vol. mm	Central Blanching	Type of skin	Length (mm) of tissue section	% No epidermis under crust	% Healing epidermis under crust	% Normal epidermis	Mono nuc. Count /mm²	PMN Count /mm²
Normal skin	-	no sulfur mustard	-	-	-	-	-	-	m	8.5 10.5	0	0	100 100	775 810	7 9
Normal skin	-	no sulfur mustard	-	-	-	-	-	-	tk	9.5 9.5	0	0	100 100	493 522	10 21
Dye Control	-	no sulfur mustard	-	-	-	-	-	-	m	9 9	0	0	100 100	817 740	5 1
Negative control A	-	sulfur mustard only	-	-	-	12x12x(0.75)	101	12x12 c/b/scab	m	12 12	32 40	44 36	24 24	577 643	15 26
Negative Control B	-	sulfur mustard only	-	-	-	12x12x(1.0)	144	12x12 c/b/scab	m	12 11.5	32 40	28 20	40 40	477 373	24 20
Control	-	(on sulfur mustard lesion)	-	-	Acetone/ AMC	13x13x(1.25)	211	8x8	m	13.5 12.5	4 4	43 42	53 54	817 740	5 1
Control	-	(on sulfur mustard lesion)	-	-	Acetone/ AMC	17x17x(1.5)	434	10x10	m	10.5 12.5	23 15	41 23	36 38	844 768	33 53
Control	-	(on sulfur mustard lesion)	-	-	Saline/ AMC	15x15x(1.5)	338	8x8	m	9.5 9.5	30 20	50 60	20 20	722 696	98 78
Control	-	(on sulfur mustard lesion)	-	-	Saline/ AMC	16x16x(1.75)	448	9x9 c/b	m	9.5 8	35 35	50 47	15 18	811 810	171 138
Control	-	(on sulfur mustard lesion)	-	-	0.05ml DMSO/AMC	18x18x(1.25)	405	10x10	m,tk	12.5 13.5	58 51	1 9	32 40	719 703	124 126
Control	-	(on sulfur mustard lesion)	-	-	0.05ml DMSO/AMC	18x18x(1.5)	486	10x10 c/b	m	10 10.5	19 25	57 48	24 27	543 518	59 46
Control	-	(on sulfur mustard lesion)	-	-	1ml DMSO/AMC	17x17x(2.5)	723	6x6	tk	10.5 10	36 28	41 48	23 24	747 720	132 113
Control	-	(on sulfur mustard lesion)	-	-	1ml DMSO/AMC	21x21x(1.25)	551	4x4 c/b	m	11 11	61 74	17 9	22 17	660 659	40 34
A-64077 (Zileuton)	Abbott	5-lipoxygenase inhibitor	1	0.1	0.05ml DMSO/AMC	15x15x(1.25)	281	8x8	tk	12.5 13.5	38 43	31 25	31 32	538 495	249 254
A-64077 (Zileuton)	Abbott	5-lipoxygenase inhibitor	10	1	0.05ml DMSO/AMC	18x18x(1.25)	405	9x9	m	16 15.5	23 28	29 19	48 53	751 710	208 178
HWA 486 (Leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	1	0.1	Saline	17x17x(1.5)	434	10x10	m	13 13	30 45	35 50	20 20	650 758	290 345
HWA 486 (Leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	10	1	Saline	22x22x(1.0)	484	7x7	m,tk	14.5 17	41 51	32 17	27 31	712 548	246 206
A77 1726B	Hoechst AG	Active Leflunomide metabolite	1	0.1	Saline	20x20x(2.0)	800	12x12	tk	13.5 14	21 45	61 38	18 17	440 418	117 111
A77 1726B	Hoechst AG	Active Leflunomide metabolite	10	1	Saline	16x16x(2.25)	576	12x12	tk	14.5 12.5	37 42	43 50	20 8	357 404	62 95
ETH 615-139	Leo, Inc.	Inhibitor of leukotriene synthesis Inhibits IL-8 gene expression	200	20	1ml DMSO/AMC	17x17x(1)	289	11x11	m	12 11.5	48 45	36 38	16 17	469 477	85 74
NPC 15669	Scios Nova, Inc	leukocyte recruitment inhibitor (an active leumedin)	2.5	0.25	Saline	17x17x(1.25)	361	9x9	m	12.5 12	15 12	46 52	39 36	647 597	98 67
NPC 14692	Scios Nova, Inc.	Negative "leumedin" control	2.5	0.25	Saline	22x22x(1.25)	605	10x10 c/b	m	12.5 12.5	27 27	27 27	46 46	1029 1061	274 318
Way-121,520	Wyeth-Ayerst	Phospholipase A_2 inhibitor Lipoxygenase inhibitor	1	0.1	Acetone	15x15x(1.5)	338	10x10 c/b	tk	10.5 12	55 56	18 24	27 20	471 683	404 380
3-Isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	1	0.1	Saline	20x20x(1.25)	500	10x10 c/b	m	11.5 9.5	21 20	46 50	33 30	664 643	200 185
3-Isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	10	1	Saline	15x15x(0.75)	169	8x8	m						

AMC = Acid Mantle Cream: 1ml
c/b = crust/blanch
m = medium
tk = thick

Table #2: Effects of Inhibitors Injected Directly into Sulfur Mustard Lesions Twice Daily for 3 Days (Rabbit #2)

Inhibitor Name	Company	Description	Application	[ ] in mg/ml	Dosage (mg)	Solvent	Lesion Size x (Thickness) in mm	Vol. mm^3	Central Blanching	Crust	Type of skin	Length (mm) of tissue section	% No epi under crust	% Healing epidermis under crust	% Normal epidermis	Mono nuc. Count /mm^2	PMN Count /mm^2
(Control)	-	(on sulfur mustard lesion)	T	-	-	DMSO	20x15x(2)	600	none	12x12	th	12 11	9 7	13 5	78 89	414 625	58 85
(Control)	-	(on sulfur mustard lesion)	IDI	-	-	DMSO	14x14x(.75)	147	D6x6	11x11	m	12 12	33 38	17 19	50 43	717 559	103 102
(Control)	-	(on sulfur mustard lesion)	IDI	-	-	PBS	17x17x(1.75)	506	none	6x6	th	16 18	31 31	11 17	58 53	49 34	151 97
(Control)		(on sulfur mustard lesion)	IDI	-	-	PBS	10x10x(1.5)	150	none	6x6 NS	th	-	-	-	-	-	-
(Control)	-	(on sulfur mustard lesion)	IDI	-	-	Saline	16x16x(.5)	128	none	9x3 NP	th	9 11	19 9	5 27	76 63	542 402	87 87
A-64077 (Zileuton)	Abbott	5-lipoxygenase inhibitor	T	0.28	0.028	DMSO	21x20x(1.5)	630	none	none	th	15 14	21 22	10 11	69 67	559 514	62 83
A-64077 (Zileuton)	Abbott	5-lipoxygenase inhibitor	IDI	0.28	0.028	DMSO	14x14x(1.25)	245	D6x6	11x11	m	-	-	-	-	-	-
HWA 486 (Leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	IDI	1	0.1	DMSO	14x14x(.75)	147	D6x6	11x11	m	-	-	-	-	-	-
HWA 486 (Leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	T	10	1	DMSO	21x21x(1.75)	772	none	11x11	th	22 24	23 23	17 24	60 53	599 636	134 131
HWA 486 (Leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	IDI	10	1	DMSO	14x14x(1)	196	D6x6	6x6	th	-	-	-	-	-	-
A77 1726B	Hoechst AG	Active Leflunomide metabolite	IDI	1	0.1	Saline	19x19x(2)	252	none	11x11	m	13 15	35 32	4 8	61 58	563 636	84 163
A77 1726B	Hoechst AG	Active Leflunomide metabolite	IDI	10	1	Saline	14x14x(1.75)	343	D6x6	8x8	th	19 12	28 26	27 21	45 53	614 511	64 73
ETH 615-139	Leo, Inc.	Inhibitor of leukotriene synthesis Inhibits IL-8 gene expression	T	15	1.5	DMSO	19x19x(2)	722	none	11x11	th	10 19	8 8	12 16	80 76	720 740	53 60
ETH 615-139	Leo,Inc.	Inhibitor of leukotriene synthesis Inhibits IL-8 gene expression	IDI	15	1.5	DMSO	14x14x(1.5)	294	D6x6	none	th	-	-	-	-	-	-
NPC 15669	Scios Nova, Inc.	leukocyte recruitment inhibitor (an active leumedin)	IDI	2.5	0.25	Saline	14x14x(2.5)	490	5x5	8x8	th	14 27	70 57	10 4.5	20 13	376 425	127 145
NPC 14692	Scios Nova, Inc.	Negative "leumedin" control	IDI	2.5	0.25	Saline	9x9x(1.5)	122	none	9x9 partNP	th	21 29	82 88	18 12	0 0	738 370	106 59
Way-121,520	Wyeth-Ayerst	Phospholipase A_2 inhibitor Lipoxygenase inhibitor	T	1	0.1	DMSO	21x21x(1.5)	662	none	10x10	th/m	12 19	22 17	7 10	72 73	661 294	24 31
Way-121,520	Wyeth-Ayerst	Phospholipase A_2 inhibitor Lipoxygenase inhibitor	IDI	1	0.1	DMSO	19x19x(1.75)	632	D 7x73 SM 13x13	none	th	-	-	-	-	-	-
3-isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	IDI	1	0.1	DMSO	13x13x(2)	338	D3x3	none	th	-	-	-	-	-	-
3-isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	T	10	1	DMSO	17x17x(1.75)	506	none	11x11	th	10 23	8 4	0 12	92 83	563 391	45 44
3-isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	IDI	10	1	DMSO	15x15x(2)	450	D6x6	2x2 NP	th	-	-	-	-	-	-
L-663,536	Merk-Frosst	Leukotriene inhibitor	IDI	0.2	0.02	PBS	14x14x(2)	392	none	1x6 NP	th	16 24	28 17	14 28	58 55	1153 527	192 42
L-656,224	Merk-Frosst	Leukotriene inhibitor	IDI	0.2	0.02	PBS	16X16X(1)	256	D7x7	6x2NP	th	-	-	-	-	-	-
IL-1ra	Synergen	IL-1 inhibitor	IDI	2	0.2	PBS	14x14x(2)	392	slight	9x5	th	13 25	31 31	6 22	63 48	548 491	80 37

IDI = Intradermal injection
T = Topical
m = medium
th = thin
PBS = phosphate buffered saline (0.01M)
** = Discontinuation of trial after first application.

NP = crust is due to needle pricks
D = due to DMSO
SM = due to sulfur mustard

Table # 3: Effects of Inhibitors Injected Directly into Sulfur Mustard Lesions Twice Daily for 3 Days (Rabbit #3)

Inhibitor Name	Company	Description	Application	[ ] in mg/ml	Dosage (mg)	Solvent	Lesion Size x (Thickness) in mm	Vol. mm^3	Central Blanching	Crust	Type of skin	Length (mm) of tissue section	% No epi under crust	% Healing epidermis under crust	% Normal epidermis	Mono nuc. Count /mm^2	PMN Count /mm^2
(Control)	-	(on sulfur mustard lesion)	T	-	-	DMSO	20x15x(2)	600	none	12x12	th	12 11	9 7	13 5	78 89	414 625	58 85
(Control)	-	(on sulfur mustard lesion)	IDI	-	-	DMSO	14x14x(.75)	147	D6x6	11x11	m	12 12	33 38	17 19	50 43	717 559	103 102
(Control)	-	(on sulfur mustard lesion)	IDI	-	-	PBS	17x17x(1.75)	506	none	6x6	th	16 18	31 31	11 17	58 53	49 34	151 97
(Control)		(on sulfur mustard lesion)	IDI	-	-	PBS	10x10x(1.5)	150	none	6x6 NP	th	-	-	-	-	-	-
(Control)	-	(on sulfur mustard lesion)	IDI	-	-	Saline	16x16x(.5)	128	none	9x3 NP	th	9 11	19 9	5 27	78 63	542 402	87 87
A-64077 (Zileuton)	Abbott	5-lipoxygenase inhibitor	T	0.28	0.028	DMSO	21x20x(1.5)	630	none	none	th	15 14	21 22	10 11	69 67	559 514	62 83
A-64077 (Zileuton)	Abbott	5-lipoxygenase inhibitor	IDI	0.28	0.028	DMSO	14x14x(1.25)	245	D6x6	11x11	m	-	-	-	-	-	-
HWA 486 (Leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	IDI	1	0.1	DMSO	14x14x(.75)	147	D6x6	11x11	m	-	-	-	-	-	-
HWA 486 (Leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	T	10	1	DMSO	21x21x(1.75)	772	none	11x11	th	22 24	23 23	17 24	60 53	599 636	134 131
HWA 486 (Leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	IDI	10	1	DMSO	14x14x(1)	196	D6x6	6x6	th	-	-	-	-	-	-
A77 1726B	Hoechst AG	Active Leflunomide metabolite	IDI	1	0.1	Saline	19x19x(2)	252	none	11x11	m	13 15	35 32	4 8	61 58	563 636	84 163
A77 1726B	Hoechst AG	Active Leflunomide metabolite	IDI	10	1	Saline	14x14x(1.75)	343	D6x6	8x8	th	19 12	28 26	27 21	45 53	614 511	64 73
ETH 615-139	Leo, Inc.	Inhibitor of leukotriene synthesis Inhibits IL-8 gene expression	T	15	1.5	DMSO	19x19x(2)	722	none	11x11	th	10 19	8 8	12 16	80 76	720 740	53 60
ETH 615-139	Leo,Inc.	Inhibitor of leukotriene synthesis Inhibits IL-8 gene expression	IDI	15	1.5	DMSO	14x14x(1.5)	294	D6x6	none	th	-	-	-	-	-	-
NPC 15669	Scios Nova, Inc.	leukocyte recruitment inhibitor (an active leumedin)	IDI	2.5	0.25	Saline	14x14x(2.5)	490	5x5	8x8	th	14 27	70 57	10 4.5	20 13	376 425	127 145
NPC 14692	Scios Nova, Inc.	Negative "leumedin" control	IDI	2.5	0.25	Saline	9x9x(1.5)	122	none	9x9 partNP	th	21 29	82 88	18 12	0 0	738 370	106 59
Way-121,520	Wyeth-Ayerst	Phospholipase A_2 Inhibitor Lipoxygenase Inhibitor	T	1	0.1	DMSO	21x21x(1.5)	662	none	10x10	th/m	12 19	22 17	7 10	72 73	661 294	24 31
Way-121,520	Wyeth-Ayerst	Phospholipase A_2 Inhibitor Lipoxygenase Inhibitor	IDI	1	0.1	DMSO	19x19x(1.75)	632	D 7x73 SM 13x13	none	th	-	-	-	-	-	-
3-Isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	IDI	1	0.1	DMSO	13x13x(2)	338	D3x3	none	th	-	-	-	-	-	-
3-Isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	T	10	1	DMSO	17x17x(1.75)	506	none	11x11	th	10 23	8 4	0 12	92 83	563 391	45 44
3-Isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	IDI	10	1	DMSO	15x15x(2)	450	D6x6	2x2 NP	th	-	-	-	-	-	-
L-663,536	Merk-Frosst	Leukotriene inhibitor	IDI	0.2	0.02	PBS	14x14x(2)	392	none	1x6 NP	th	16 24	28 17	14 28	58 55	1153 527	192 42
L-656,224	Merk-Frosst	Leukotriene inhibitor	IDI	0.2	0.02	PBS	16X16X(1)	256	D7x7	6x2NP	th	-	-	-	-	-	-
IL-1ra	Synergen	IL-1 inhibitor	IDI	2	0.2	PBS	14x14x(2)	392	slight	9x5	th	13 25	31 31	6 22	63 48	546 491	80 37

IDI = Intradermal injection
T = Topical
m = medium
th = thin
PBS = phosphate buffered saline (0.01M)
** = Discontinued of trial after first application.

NP = crust is due to needle pricks
D = due to DMSO
SM = due to sulfur mustard

Table #4: Effects of Inhibitors Injected Directly into Sulfur Mustard Lesions Twice Daily for 4 Days (Rabbit #4)

Inhibitor Name	Company	Description	Application	[ ] in mg/ml	Dosage (mg)	Solvent	Lesion Size x (Thickness) in mm	Vol. mm³	Central Blanching	Crust	Length (mm) of tissue section	% No epi under crust	% Healing epidermis under crust	% Normal epidermis	Mono nuc. Count /mm²	PMN Count /mm²
(Normal Skin)	-	(no sulfur mustard)	ID	-	-	5% DMSO/ in Saline	0x0x(0)	0	none	none	- -	0 0	0 0	100 100	1031 1312	24 31
(Normal Skin)	-	(no sulfur mustard)	ID	-	-	5% DMSO/ in Saline	0x0x(0)	0	none	none	- -	0 0	0 0	100 100	532 504	10 4
(Negative Control)	-	(sulfur mustard only)	-	-	-	-	12x12x(0.75)	108	10x10	-	12.5 15	42 32	35 32	23 36	687 619	440 199
(Negative Control)	-	(sulfur mustard only)	-	-	-	-	13x13x(1.25)	211	-	12x12	14.5 14.5	37 27	55 70	8 3	585 539	166 104
(Control)	-	(on sulfur mustard lesion)	T	-	-	AMC/ Saline	12x12x(1.5)	216	9x9	-	14.5 14.5	23 20	50 53	27 27	836 1152	748 939
(Control)	-	(on sulfur mustard lesion)	T	-	-	AMC/ DMSO	12x12x(1)	144	10x10	-	9.5 10.5	42 50	38 50	20 0	1019 1026	436 488
(Control)	-	(on sulfur mustard lesion)	ID	-	-	5%DMSO / Saline	10x10x(0.75)	75	10x10	10x10	11.5 11.5	54 63	29 21	17 17	651 394	81 92
(Control)	-	(on sulfur mustard lesion)	ID	-	-	5%DMSO / Saline + Tween	13x13x(2)	338	-	10x10	14.5 15.5	83 88	10 6	7 6	692 647	150 105
A-64077 (Zileuton)	Abbott	5-lipoxygenase	ID	0.7	0.35	5%DMSO /Saline	12x12x(1.5)	216	-	8x8	13 15.5	48 50	41 37	11 13	1000 919	253 219
L-656,224	Merk-Frosst	Leukotriene inhibitor	ID	0.5	0.05	5%DMSO /Saline	12x12x(1.75)	252	-	10x10	16 14.5	47 54	38 23	15 23	639 638	471 451
ETH615-139	Leo, Inc	Inhibitor of leukotriene synthesis Inhibits IL-8 gene expression	ID	7.5	0.75	10%DMSO /Saline	16x16x(3.5)	896	-	13x13	• •	• •	• •	• •	• •	• •
3-isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	ID	2.5	0.25	5%DMSO /Saline + Tween	13x13x(1.25)	211	-	9x9	12.5 12.5	21 21	79 79	0 0	801 779	190 160
ETH615-139	Leo, Inc.	Inhibitor of leukotriene synthesis Inhibits IL-8 gene expression	T	200	20	AMC/ DMSO	11x11x(1)	121	11x11	11x11	11.5 10.5	46 50	16 5	38 45	681 810	49 86
3-isobutyl-1-methyl xanthine	Sigma	Cyclic AMP phosphodiesterase inhibitor	T	10	1.0	AMC/ DMSO	13x13x(1.75)	296	-	10x10	13.5 12.5	28 35	43 38	29 27	1085 1094	814 757
HWA 486 (leflunomide)	Hoechst AG	Inhibitor of macrophage and lymphocyte proliferation	T	20	2.0	AMC/ DMSO	11x11x(1.5)	182	6x6	6x6	14.5 14.5	40 33	40 40	20 27	702 682	797 738
A771726B	Hoechst AG	Active Leflunomide metabolite	T	20	2.0	AMC/ DMSO	13x13x(1.25)	211	10x10	-	14 14.5	48 50	52 43	0 7	861 886	672 719
NPC-14692	Scios Nova, Inc.	Negative "leumedin" control	T	10	1.0	AMC/ Water	13x13x(1.25)	211	7x7	-	12.5 12.5	58 54	34 38	8 8	1468 775	1199 669
NPC-15669	Scios Nova, Inc.	Leukocyte recruitment inhibitor (an active leukomedin)	T	10	1.0	AMC/ Water	14x14x(1)	196	14x14	14x14	7.5 8.5	25 22	62 56	13 22	1072 830	468 380
NPC-14692	Scios Nova, Inc.	Negative "leumedin" control	ID	15	1.5	Water	16x16x(1.5)	384	-	12x12	• •	• •	• •	• •	• •	• •
NPC-15669	Scios Nova, Inc.	Leukocyte recruitment inhibitor (an active leukomedin)	ID	15	1.5	Water	15x15x(2.5)	563	-	10x10	• •	• •	• •	• •	• •	• •
Way-121,520-4	Wyeth-Ayerst	Phospholipase A_2 inhibitor Lipoxygenase inhibitor	T	5	0.5	AMC/ DMSO	12x12x(0.75)	108	10x10	10x10	14.5 13.5	50 53	30 25	20 22	1056 959	519 401
L-663,536	Merk-Frosst	Leukotriene inhibitor	ID	1	0.1	5%DMSO /Saline	12x12x(1.25)	180	-	9x9	• •	• •	• •	• •	• •	• •
IL-1ra	Synergen	IL-1 inhibitor	T	4	0.4	AMC/ PBS	16x16x(1.75)	252	-	15x15	14.5 15.5	37 25	30 37	33 38	1231 1232	802 791
L-656,224	Merk-Frosst	Leukotriene inhibitor	T	2	0.2	5%DMSO /AMC	12x12x(1.25)	180	8x8	8x8	12.5 12.5	35 35	57 50	8 15	1691 1237	1680 1210
L-663,536	Merk-Frosst	Leukotriene inhibitor	T	2	0.2	5%DMSO /AMC	14x14x(2)	392	-	11x11	9.5 -	90 -	10 -	0 -	991 -	417 -

All lesions were thin slices
PBS = phosphate buffered saline (0.01M)
T = Topical
AMC = Acid Mantle Cream
• = results could not be attained

Table #5: Effects of Inhibitors Injected Directly into Sulfur Mustard Lesions Twice Daily for 3 Days (Rabbit #5)

Inhibitor Name	Company	Description	[ ] in mg/ml	Dosage (mg)	Solvent	Lesion Size x (Thickness) in mm	Vol. mm^3	Central Blanching	Crust	Type of skin	Length (mm) of tissue section	% No epi under crust	% Healing epidermis under crust	% Normal epidermis	Mono nuc. Count /mm^2	PMN Count /mm^2
(Normal Skin)	-	(no sulfur mustard)	-	-	-	-	0	none	none		- -	0 0	0 0	100 100	752 839	5 1
(Negative Control)	-	(sulfur mustard only)	-	-	-	12x12x(2)	288	none	10x10		10.5 11	73 65	9 17	18 18	864 935	179 161
(Negative Control)	-	(sulfur mustard only)	-	-	-	14x14x(2)	392	none	12x12		15.5 15.5	62 62	25 31	13 7	973 927	922 762
(Negative Control)	-	(sulfur mustard only)	-	-	-	12x12x(1.5)	216	none	10x10		7.5 8.5	100 100	0 0	0 0	1345 1136	154 146
(Control)	-	(on sulfur mustard lesion)	-	-	Saline	10x10x(1.25)	125	none	9x9		6.5 6.5	100 100	0 0	0 0	823 844	99 81
(Control)	-	(on sulfur mustard lesion)	-	-	5%DMSO / Saline + Tween	12x12x(2.25)	324	none	10x10		9.5 9.5	75 70	25 30	0 0	748 785	145 245
(Control)	-	(on sulfur mustard lesion)	-	-	PBS	12x12x(2)	288	none	10x10		9.5 9.5	100 100	0 0	0 0	1172 1109	242 181
(Control)	-	(on sulfur mustard lesion)	-	-	5%DMSO / Saline	15x11x(1.75)	289	none	13x9		10.5 11.5	86 92	14 8	0 0	817 529	108 133
sTNF	Immunex	Soluble Human TNF Receptor	0.1	0.01	Saline	13x13x(2)	338	11x11	10x10		12.5 11.5	88 87	12 13	88 87	890 694	279 175
sTNF	Immunex	Soluble Human TNF Receptor	0.1	0.01	Saline	12x12x(2.5)	360	10x10	8x8		9.5 9	65 65	25 35	0 0	743 742	179 249
sTNF	Immunex	Soluble Human TNF Receptor	0.4	0.04	Saline	12x12x(1.25)	180	10x10	4x8		10.5 9.5	73 75	27 25	0 0	623 710	246 234
sTNF	Immunex	Soluble Human TNF Receptor	0.4	0.04	Saline	11x11x(2)	242	9x9	6x6		8.5 8.5	50 45	17 22	33 33	106 624	330 320
sIL1	Immunex	Soluble Human IL1 Receptor	0.1	0.01	Saline	12x12x(1.75)	252	9x9	8x8		9.5 10.5	70 77	30 23	0 0	760 1005	266 17
sIL1	Immunex	Soluble Human IL1 Receptor	0.1	0.01	Saline	15x15x(2)	450	11x11	8x4		8.5 8	72 65	28 35	0 0	1080 1130	211 321
sIL1	Immunex	Soluble Human IL1 Receptor	0.4	0.04	Saline	14x14x(3)	588	12x12	10x10		11.5 10.5	58 55	17 18	25 27	768 998	638 663
sIL1	Immunex	Soluble Human IL1 Receptor	0.4	0.04	Saline	17x17x(3.5)	1012	13x13	13x13		12 12	76 76	4 12	20 12	675 795	678 906
sCR1	T Cell Sciences	Soluble Human Complement Receptor	0.58	0.058	Saline	14x14x(3)	588	none	11x11		11.5 10.5	88 91	12 9	0 0	909 781	536 441
sCR1	T Cell Sciences	Soluble Human Complement Receptor	0.58	0.058	Saline	14x14x(2)	392	11x11	6x6		10.5 9.5	82 90	18 10	0 0	736 670	161 214
sCR1	T Cell Sciences	Soluble Human Complement Receptor	2.9	0.29	Saline	14x11x(3)	462	none	13x9		9.5 8.5	90 94	10 6	0 0	441 347	282 381
sCR1	T Cell Sciences	Soluble Human Complement Receptor	2.9	0.29	Saline	12x12x(2)	288	10x10	9x9		11.5 11.5	67 67	16 16	17 17	1032 1152	694 180
Pentoxifylline	Hoecht-Roussel	Hemorheologic Agent	1	0.1	Saline	12x16x(3)	576	none	5x11		11.75 11.5	67 67	17 16	16 17	623 798	335 457
Pentoxifylline	Hoecht-Roussel	Hemorheologic Agent	1	0.1	Saline	14x14x(2)	392	13x11	8x8		9.5 9.5	60 65	30 25	10 10	810 973	178 165
Pentoxifylline	Hoecht-Roussel	Hemorheologic Agent	10	1	Saline	14x14x(3)	588	12x12	10x10		11 11.5	70 71	17 8	13 21	655 679	208 277
Pentoxifylline	Hoecht-Roussel	Hemorheologic Agent	10	1	Saline	14x14x(2.5)	480	12x12	8x8		11 10.5	70 77	13 14	17 9	795 694	709 545
IL1-ra	Synergen	IL-1 inhibitor	10	1	PBS	14x14x(1.25)	245	11x11	8x8		10.5 10.5	73 77	9 14	18 9	699 713	313 183
IL-1ra	Synergen	IL-1 inhibitor	10	1	PBS	11x11x(1)	121	10x10	8x8		9.5 8.5	60 72	20 6	20 22	555 128	105 154
L-663,536			5	0.5	5%DMSO /Saline	14x14x(3)	588	11x11	5x5							
3-isobutyl-1-methyl Xanthine			5	0.5	5%DMSO /Saline + Tween	15x15x(3)	675	none	11x11		11 12.5	74 85	17 7	9 8	918 986	323 261
3-isobutyl-1-methyl Xanthine			5	0.5	5%DMSO /Saline + Tween	12x13x(3)	468	none	10x10		11.5 11.5	79 75	13 17	8 8	661 543	356 449

Table #6: Effects of Inhibitors Injected Directly into Sulfur Mustard Lesions Twice Daily for 3 Days (Rabbit #6)

#	Inhibitor Name	Company	Description	[ ] in mg/ml	Dosage (mg)	Solvent	Lesion Size x (Thickness) in mm	Vol. mm³	Central Blanching	Crust	Type of skin	Length (mm) of tissue section	% No epi under crust	% Healing epidermis under crust	% Normal epidermis	Mono nuc. Count /mm²	PMN Count /mm²
	(Normal Skin)	-	(no sulfur mustard)	-	-	-	-	0	none	none		- -	0 0	0 0	100 100	752 839	5 1
NCa	(Negative Control)	-	(sulfur mustard only)	-	-	-	12x12x(2)	288	none	10x10		10.5 11	73 65	9 17	18 18	864 935	179 161
NCb	(Negative Control)	-	(sulfur mustard only)	-	-	-	14x14x(2)	392	none	12x12		15.5 15.5	62 62	25 31	13 7	973 927	922 762
NCc	(Negative Control)	-	(sulfur mustard only)	-	-	-	12x12x(1.5)	216	none	10x10		7.5 8.5	100 100	0 0	0 0	1345 1136	154 146
1	(Control)	-	(on sulfur mustard lesion)	-	-	Saline	10x10x(1.25)	125	none	9x9		6.5 6.5	100 100	0 0	0 0	823 844	99 81
2	(Control)	-	(on sulfur mustard lesion)	-	-	5%DMSO / Saline + Tween	12x12x(2.25)	324	none	10x10		9.5 9.5	75 70	25 30	0 0	748 785	145 245
3	(Control)	-	(on sulfur mustard lesion)	-	-	PBS	12x12x(2)	288	none	10x10		9.5 9.5	100 100	0 0	0 0	1172 1109	242 181
4	(Control)	-	(on sulfur mustard lesion)	-	-	5%DMSO / Saline	15x11x(1.75)	289	none	13x9		10.5 11.5	86 92	14 8	0 0	817 529	108 133
5a	sTNF	Immunex	Soluble Human TNF Receptor	0.1	0.01	Saline	13x13x(2)	338	11x11	10x10		12.5 11.5	88 87	12 13	88 87	890 694	279 175
5b	sTNF	Immunex	Soluble Human TNF Receptor	0.1	0.01	Saline	12x12x(2.5)	360	10x10	8x8		9.5 9	65 65	25 35	0 0	743 742	179 249
6a	sTNF	Immunex	Soluble Human TNF Receptor	0.4	0.04	Saline	12x12x(1.25)	180	10x10	4x8		10.5 9.5	73 75	27 25	0 0	623 710	246 234
6b	sTNF	Immunex	Soluble Human TNF Receptor	0.4	0.04	Saline	11x11x(2)	242	9x9	6x6		8.5 8.5	50 45	17 22	33 33	106 624	330 320
7a	sIL1	Immunex	Soluble Human IL1 Receptor	0.1	0.01	Saline	12x12x(1.75)	252	9x9	8x8		9.5 10.5	70 77	30 23	0 0	760 1005	266 17
7b	sIL1	Immunex	Soluble Human IL1 Receptor	0.1	0.01	Saline	15x15x(2)	450	11x11	8x4		8.5 8	72 65	28 35	0 0	1080 1130	211 321
8a	sIL1	Immunex	Soluble Human IL1 Receptor	0.4	0.04	Saline	14x14x(3)	588	12x12	10x10		11.5 10.5	58 55	17 18	25 27	768 998	638 663
8b	sIL1	Immunex	Soluble Human IL1 Receptor	0.4	0.04	Saline	17x17x(3.5)	1012	13x13	13x13		12 12	76 76	4 12	20 12	675 795	678 906
9a	sCR1	T Cell Sciences	Soluble Human Complement Receptor	0.58	0.058	Saline	14x14x(3)	588	none	11x11		11.5 10.5	88 91	12 9	0 0	909 781	536 441
9b	sCR1	T Cell Sciences	Soluble Human Complement Receptor	0.58	0.058	Saline	14x14x(2)	392	11x11	6x6		10.5 9.5	82 90	18 10	0 0	736 670	161 214
10a	sCR1	T Cell Sciences	Soluble Human Complement Receptor	2.9	0.29	Saline	14x11x(3)	462	none	13x9		9.5 8.5	90 94	10 6	0 0	441 347	282 381
10b	sCR1	T Cell Sciences	Soluble Human Complement Receptor	2.9	0.29	Saline	12x12x(2)	288	10x10	9x9		11.5 11.5	67 67	16 16	17 17	1032 1152	694 180
11a	Pentoxifylline	Hoecht-Roussel	Hemorheologic Agent	1	0.1	Saline	12x16x(3)	576	none	5x11		11.75 11.5	67 67	17 16	16 17	623 798	335 457
11b	Pentoxifylline	Hoecht-Roussel	Hemorheologic Agent	1	0.1	Saline	14x14x(2)	392	13x11	8x8		9.5 9.5	60 65	30 25	10 10	810 973	178 165
12a	Pentoxifylline	Hoecht-Roussel	Hemorheologic Agent	10	1	Saline	14x14x(3)	588	12x12	10x10		11 11.5	70 71	17 8	13 21	655 679	208 277
12b	Pentoxifylline	Hoecht-Roussel	Hemorheologic Agent	10	1	Saline	14x14x(2.5)	480	12x12	8x8		11 10.5	70 77	13 14	17 9	795 694	709 545
13a	IL1-ra	Synergen	IL-1 Inhibitor	10	1	PBS	14x14x(1.25)	245	11x11	8x8		10.5 10.5	73 77	9 14	18 9	699 713	313 183
13b	IL-1ra	Synergen	IL-1 Inhibitor	10	1	PBS	11x11x(1)	121	10x10	8x8		9.5 8.5	60 72	20 6	20 22	555 128	105 154
14	L-663,536			5	0.5	5%DMSO /Saline	14x14x(3)	588	11x11	5x5							
15a	3-isobutyl-1-methyl Xanthine			5	0.5	5%DMSO /Saline + Tween	15x15x(3)	675	none	11x11		11 12.5	74 85	17 7	9 8	918 986	323 261
15b	3-isobutyl-1-methyl Xanthine			5	0.5	5%DMSO /Saline + Tween	12x13x(3)	468	none	10x10		11.5 11.5	79 75	13 17	8 8	661 543	356 449